Received: 2 July 2019 | Revised: 21 September 2019 | Accepted: 21 October 2019

DOI: 10.1002/cche.10234

CEREALS & GRAINS ASSOCIATION

RESEARCH ARTICLE

Effect of fermentation time on the nutritional properties of pea protein-enriched flour fermented by *Aspergillus oryzae* and *Aspergillus niger*

Hayley M. Kumitch[1] | **Andrea Stone**[1] | **Matthew G. Nosworthy**[2] | **Michael T. Nickerson**[1] | **James D. House**[2,3,4] | **Darren R. Korber**[1] | **Takuji Tanaka**[1]

[1]Department of Food and Bioproduct Sciences, University of Saskatchewan, Saskatoon, SK, Canada

[2]Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada

[3]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB, Canada

[4]Canadian Centre for Agri-Food Research in Health and Medicine, University of Manitoba, Winnipeg, MB, Canada

Correspondence
Takuji Tanaka, Department of Food and Bioproduct Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK S7N 5A8, Canada.
Email: Takuji.Tanaka@usask.ca

Funding information
saskatchewan ministry of agriculture, Grant/Award Number: Agriculture Development Fund #20140283; Global Institute for Food Security, Saskatoon, SK

Abstract

Background and objectives: The overall goal of this research was to examine the effect of solid-state fermentation using *Aspergillus oryzae* and *Aspergillus niger* over time on the nutritional properties of an air-classified pea protein-enriched flour (PPEF). Specifically, changes to levels of enzyme inhibitors, total phenolics, in vitro protein digestibility (IVPD), and protein quality (IVPD-corrected amino acid score) were assessed.

Findings: Trypsin and chymotrypsin inhibitors were reduced by 11.6 and 30.4%, and 22.8 and 21.8% for *A. niger* and *A. oryzae*, respectively, whereas the total phenolic content increased by 35.3 and 44.7% for *A. niger* and *A. oryzae*, respectively. IVPD increased over the fermentation time. However, the overall protein quality as measured by the IVPD-corrected amino acid score became worse, becoming lowered by 5%–15% after 6 hr.

Conclusions: SSF improved the digestibility and bioactive compound availability, whereas protein quality was not improved.

Significance and novelty: Findings indicate that this particular solid-state fermentation system improved the digestibility of the PPEF.

KEYWORDS

bioactive compounds, pea protein-enriched flour, protein quality, solid-state fermentation

1 | INTRODUCTION

Pea protein ingredients are becoming increasingly popular as an alternative protein source to soy, wheat, or animal-derived proteins because of their low cost, abundance, nongenetically modified status, and nutritional status (Meinlschmidt, Ueberham, Lehmann, Schweiggert-Weisz, & Eisner, 2005). Peas are high in protein, carbohydrates, vitamins, and minerals, but low in fat (Boye, Zare, & Pletch, 2010). Commercially, pea protein ingredients are available in flours, as an air-classified protein-enriched flour, or as concentrates or isolates prepared from a wet extraction process (Schutyser, Pelgrom, van der Goot, & Boom, 2015). Increased utilization of the air-classified protein-enriched flour is attractive due to its higher nutritional value than the flour (i.e., more protein), without the costly processing challenges associated with wastewater and drying of wet extraction (Pelgrom, Vissers, Boom, & Schutyser, 2013). The protein fractions of whole pulse seeds also contain levels of bioactive compounds that are known to have both positive and negative effects on health and protein digestion (Schutyser et al., 2015). These compounds include proteinase/amylase inhibitors, phytic acid, lectins, phenolics,

tannins, oxalates, saponins, and oligosaccharides (Deshpande et al., 2000; Sandberg, 2002). Typically, these compounds are reduced either by soaking or by thermal means; however, other methods such as fermentation have shown some promising results (Granito et al., 2002).

Trypsin and chymotrypsin inhibitors act to inhibit proteases in the digestive tract, making proteins less susceptible to digestive enzymes. The presence and levels of these compounds can have a direct effect on protein digestibility. Phenolic compounds are known to exhibit antioxidant activities, and the change in their contents has been demonstrated to improve health-linked functionality (Torino et al., 2013). Protein quality is related to the composition of essential amino acids, digestibility, and bioavailability (Millward, Layman, Tome, & Schaafsma, 2008). Bioavailability may be defined as the proportion of ingested dietary amino acids that are absorbed in a chemical form suitable for utilization for metabolic processes in our body. Cuevas-Rodriguez, Milan-Carrillo, Mora-Escobedo, Cardenas-Valenzuela, and Reyes-Moreno (2004) reported that maize tempeh flour protein quality increased through fungal solid-state fermentation (SSF) in which the true protein content and in vitro protein digestibility increased. This was attributed to the reduction in bioactive compounds, protein denaturation during cooking, and protein hydrolysis during fermentation. The authors, however, did not consider the impact of fermentation on the amino acid profile and further lacked the protein digestibility-corrected amino acid scores (PDCAASs) which is the international unit of measure for protein quality. The effect of fermentation on the nutritive compounds of a pea protein ingredient has previously been studied for submerged fermentation with *Lactobacillus plantarum* (Cabuk et al., 2018). The total phenols and tannins increased, chymotrypsin inhibitor activity decreased, and in vitro protein digestibility increased; however, the bacteria that were used metabolized the sulfur amino acids decreasing the overall protein quality; the authors concluded that fermentation with other microorganisms should be considered. Kannan, Nielsen, and Mason (2001) reported that solid-state fungal (*Rhizopus oligosporus*) fermentation (25 hr, 37°C) of cooked black beans did not impact either the true protein digestibility or PDCAAS; however, they did not study the effect of fermentation time.

We previously demonstrated that limited hydrolysis (10%–11% degree of hydrolysis) of pea protein-enriched flour (PPEF) was achieved through 6 hr of SSF with *Aspergillus niger* and *Aspergillus oryzae* and that the resultant fermented PPEF had improved water hydration and oil-holding capacities (Kumitch, 2019). In the current study, we hypothesized that utilizing the same SSF conditions could also influence the nutritional value of PPEF. Our overarching goal is to use solid-state fermentation with GRAS fungi (*A. niger* NRRL 334 and *A. oryzae* NRRL 5,590) to reduce the levels of protease inhibitors and improve the protein quality of PPEF while also measuring the impact on total phenolic content. To our knowledge, studies have been limited on the use of solid-state fermentation to improve the nutritional value of pea protein-enriched flour specifically. Value-added processing of PPEF will allow for increased utilization of pea protein in applications such as sports/nutrition bars.

Highlights

- Pea protein flour was fermented by *Aspergillus oryzae* and *Aspergillus niger*.
- SSF of pea protein is a tool to improve nutritional quality.
- SSF increased phenolic content and decreased enzyme inhibitor activities.
- The enzyme inhibitory activities were decreased through SSF.
- The in vitro protein digestibility increased from SSF.
- The limiting amino acid score decreased over the fermentation time.

2 | MATERIALS AND METHODS

2.1 | Materials

Air-classified pea protein-enriched flour (PPEF) was provided by Parrheim Foods (Saskatoon, SK, Canada), whereas *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5,590 strains were obtained from the Agriculture Research Service, US Department of Agriculture (Peoria, IL, USA). All chemicals used in this study were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada) and were of reagent grade.

2.2 | Fermentation

Fungal strains (*Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5,590) were cultivated on potato dextrose agar (PDA) and incubated at 30°C for 7 days under aerobic conditions. Spores were collected from fungal cultures fully grown in petri dishes using 10 ml of deionized water, and the concentration of spores was determined by direct microscopy counting (Leica, Model S6E, Wetzlar, Germany) using a hemocytometer (Bright-Line, Horsham, PA, USA).

PPEF (230 g) was inoculated at a rate of 10^7 spore/g substrate (1 ml aliquot). The moisture content was calculated as water weights per total weight of culture bed and was maintained at 50% (Equation 1). M_n is the moisture content (%) of the material of n, W_w is the wet weight of the sample, and W_d is the weight of the sample after drying:

$$M_n = \left(\frac{W_w - W_d}{W_w}\right) \times 100 \quad (1)$$

After mixing the dough with a kitchen mixer, it was spread on a sheet pan with a thickness of approximately 25 mm. Fermentation was conducted at 40°C in an Isotemp incubator (Fisher Scientific, Model 650D, Waltham, MA, USA) for 6 hr. Samples (80 g) were taken at times 0, 2, 4, and 6 hr for analysis. Three independent fermentation tests were conducted using separate spore suspensions for each batch. Samples were deactivated at 80°C in a water bath and suspended in deionized water adjusted to pH 8.0 before freeze-drying for 48 hr into a powder (Labconco, FreeZone 12, Kansas City, USA). The three independent fermentation tests for each time/fungi were combined to form a composite batch in order to obtain a uniform sample and eliminate any minor differences in batch-to-batch variability. The dried powder was stored at room temperature (21–23°C) for further testing.

2.3 | Bioactive properties

2.3.1 | Trypsin inhibitor activity

Trypsin inhibitory activity (TIA) was determined according to the AOAC method 22-40.01, using a UV/visible spectrophotometer (AOAC, 2005). In brief, 0.25 g of PPEF and 25 ml of 0.01 N NaOH were vortexed for 1 min in a centrifuge tube. The tube was then centrifuged at 14,000 *g* for 10 min at 4°C using a 5804R centrifuge (Eppendorf, Hamburg, Germany). The aliquots (0, 0.6, 1.0, 1.4, 1.8 ml) of the diluted supernatant were pipetted into test tubes and adjusted to 2.0 ml with deionized water. Following this, the tubes were incubated with 2 ml of trypsin solution (4 mg of trypsin in 200 ml of 0.001 M HCl) in a 37ºC water bath (VWR Scientific Products, Radnor, PA, USA) for 5 min. Five milliliters of prewarmed *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (DL-BAPNA) was added to the tubes with 1 ml of dimethyl sulfoxide and diluted by Tris buffer (0.05 M, pH 8.2) to 100 ml. The samples were incubated for 10 min, and then, 1 ml of 30% acetic acid solution was added to the solution to terminate the reaction. The samples were filtered through Whatman No. 2 paper. One trypsin inhibitor unit (TIU) is equivalent to an increase of 0.01 absorbance unit at 410 nm per 10 ml of reaction mixture compared with the blank sample. The blank was prepared for each sample concentration with acetic acid addition before the trypsin solution. The trypsin inhibitory activity was calculated using Equation 2:

$$\left(\frac{\text{TIU}}{\text{mg sample}}\right) = \left(\frac{\text{TIU}}{\text{mL of extract taken}}\right) \times \left(\frac{\text{25 mL of extract}}{\text{500 mg of sample}}\right) \times \text{DF} \times \left(\frac{100\%}{100\% - \text{MC}}\right) \quad (2)$$

where TIU is the trypsin inhibitor unit, DF is the dilution factor, and MC is the moisture content of PPEF samples. Measurements were made in triplicate, on the composite batch sample ($n = 3$), and reported as the mean ± standard deviation.

2.3.2 | Chymotrypsin inhibitor activity

The chymotrypsin inhibitory activity (CIA) was determined according to Makkar, Siddhuraju, and Becker (2007). In brief, 1 g of PPEF was placed in a 50-ml centrifuge tube with 10 ml of borate buffer (0.1 M, pH 7.6). The sample was vortexed for 1 min and then placed on a mechanical stirrer at 500 rpm for 1 hr. The sample was centrifuged at 3,000 *g* for 10 min at 4°C using a 5804R centrifuge (Eppendorf, Hamburg, Germany). In test tubes, 0, 0.25, 0.5, and 0.75 ml of sample were diluted to 1 ml with borate buffer followed by incubation at 37°C for 10 min with 1 ml of chymotrypsin stock solution (4 mg chymotrypsin in 100 ml 0.001 M HCl). Then, 2 ml of prewarmed casein solution (1 g casein in 100 ml borate buffer, pH 7.6, 37°C) was added to the solution and incubated for 10 min. The reaction was terminated by the addition of 6 ml of 18% trichloroacetic acid. Trichloroacetic acid was prepared by 18 g of anhydrous sodium acetate and 20 ml of glacial acetic acid, diluted to 1 L with deionized water. The solution was then cooled to room temperature (21–23°C) for 30 min followed by filtering through Whatman No. 2 paper. The absorbance of filtered sample was measured at 275 nm against the appropriate blank (6 ml of trichloroacetic acid and 2 ml of casein solution). The chymotrypsin unit is defined as the increase by 0.01 absorbance unit at 275 nm. Chymotrypsin inhibitory activity is defined by the number of chymotrypsin units inhibited and can be expressed as CIU per milligram of the sample, as follows (Equation 3):

$$\left(\frac{\text{CIU}}{\text{mg sample}}\right) = \left(\frac{\text{CIU}}{\text{mL of extract taken}}\right) \times \left(\frac{\text{10 mL of extract}}{\text{1000 mg of sample}}\right) \times \text{DF} \times \left(\frac{100\%}{100\% - \text{MC}}\right) \quad (3)$$

where DF is the dilution factor and MC is moisture content of PPEF samples. Measurements were made in triplicate, on the composite batch sample ($n = 3$), and reported as the mean ± standard deviation.

2.3.3 | Total phenolic content

The total phenolic compounds (TPCs) were determined according to Waterman & Mole (1994) using the Folin–Ciocalteu assay. In brief, 1 g of sample was extracted with 15 ml solvent (1% HCl in methanol) for 2 hr, followed by centrifugation for 10 min at 1,510 *g* and 25ºC to recover the supernatant. Following this, 5 ml of solvent was added to the residue after removal of supernatant and vortexed every 5 min for 20 min. This method was repeated 3 times

and the supernatants pooled for analysis. From the pooled supernatants, 0.5 ml was combined with 2.5 ml of Folin–Ciocalteu reagent. The solution was at 25ºC for 5 min before the addition of sodium carbonate solution (20% w/v) and adjusting the volume to 50 ml with deionized water. After 2 hr, the absorbance of samples was measured at 760 nm. A gallic acid standard curve was prepared using a 1 mg/ml gallic acid stock solution with diluted water to obtain concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml. Total phenolic content result was expressed as gram gallic acid equivalents per 100 g of sample on dry basis using the following equation (Equation 4):

$$\left(\frac{\text{g gallic acid equivalent}}{\text{100 g of sample}}\right) = \left(\frac{\text{ABS} - y}{a}\right) * C \quad (4)$$

where ABS is absorbance, C (mg/ml) is the concentration of the sample used, y is the y-intercept, and a is the slope of the standard curve. Measurements were made in triplicate, on the composite batch sample ($n = 3$), and reported as the mean ± standard deviation.

2.4 | Protein quality

The crude protein (Method #: 984.13A; $\%N \times 6.25$) and moisture (Method #: 925.10) contents of fermented PPEF were carried out according to the Association of Official Analytical Chemists (AOAC) methods. Amino acids, excepting methionine, cysteine, and tryptophan, were determined according to AOAC Official Method 982.30. AOAC 985.28 was used to determine methionine and cysteine content after performic acid oxidation and acid hydrolysis. The AccQ-Tag Ultra protocol (Astephen, 2018), using an AccQ-Tag Ultra C18, 1.7 μm column on a Shimadzu UPLC system, was used to determine the amino acid profiles. Tryptophan content was determined according to the ISO protocol 13,904 (International Organization for Standardization, 2016). To control for hydrolysis/derivatization conditions, samples of NIST 3,234, a soy flour standard, were hydrolyzed and analyzed alongside experimental samples. In vitro protein digestibility (enzymatic pH-drop assay) and determination of in vitro protein digestibility-corrected amino acid score (IV-PDCAAS) for all fermented PPEF samples were performed according to Bai, Nosworthy, House, and Nickerson (2018). The multienzyme solution for in vitro protein digestibility was prepared by mixing 31 mg of chymotrypsin (bovine pancreas ≥ 40 units/mg protein), 16 mg of trypsin (porcine pancreas 13,000–20,000 BAEE units/mg protein), and 13 mg of protease (*Streptomyces griseus* ≥ 15 units/mg solid) within 10 ml of deionized water. The pH of the solution was adjusted to 8.0 using 0.1 M NaOH and HCl and was stored at 37°C. PPEF sample in the amount of 62.5 ± 0.5 mg of protein was added to 10 ml of deionized water. The solution was stirred for 1 hr at 37°C and pH adjusted to pH 8.0 before adding 1 ml of the multienzyme solution. The pH of the protein solution was monitored and recorded every 1 min for 10 min, and the in vitro protein digestibility was calculated using the calculation below (Equation 5):

$$\text{IVPD} = 65.66 + 18.10 * \Delta\text{pH}_{10\text{min}} \quad (5)$$

where $\Delta\text{pH}_{10\text{min}}$ refers to the change in pH from initial 8.0 to the end of the 10 min. The amino acid score was calculated as the ratio of individual amino acids in 1 g of PPEF to the FAO-recommended reference protein standard for 2- to 5-year-old preschool children (FAO, 1991). The amino acid composition of the reference protein was as follows (amino acid, mg/g protein): histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; methionine + cysteine, 25; phenylalanine + tyrosine, 63; threonine, 34; tryptophan, 11; and valine, 35. The limiting amino acid was denoted by the lowest ratio.

2.5 | Statistics

All measurements were made in triplicate on the composite batch for each fermentation time and fungi type ($n = 3$), and reported as the mean ± standard deviation (except for the amino acid profile, which was only measured once on each composite batch). Systat 4.0 (Systat Software, San Jose, CA, USA) was used to complete the statistical tests. A two-way analysis of variance (ANOVA) was used to test for differences within the main effects of fermentation time and the fungi type, and their associated interaction. A one-way ANOVA and Scheff's post hoc test were utilized to determine the statistical significance ($p < .05$) for each fungi type for the bioactive properties and IVPD.

3 | RESULTS AND DISCUSSION

3.1 | Bioactive compounds

Trypsin and chymotrypsin are serine proteolytic enzymes that aid in digestion of proteins. Trypsin cleaves lysine and arginine residues, whereas chymotrypsin cleaves at hydrophobic residues such as tyrosine, tryptophan, and phenylalanine on polypeptide chains. However, trypsin and chymotrypsin inhibitors are able to form stable complexes with the enzymes, inhibiting trypsin/chymotrypsin from binding to the protein substrates resulting in the reduced digestibility of proteins; thus, a decrease in TIA or CIA would serve to increase the quality of protein materials (Shi, Mu, Arntfield, & Nickerson, 2017). Fermentation of PPEF was evaluated in respect to the levels of TIA and CIA over the duration of fermentation (Table 1). Both TIA and CIA of fermented PPEF had a statistically significant interaction

TABLE 1 Bioactive properties of fermented PPEF

Fermentation time (h)	TIA (TIU/mg)	CIA (CIU/mg)	TPC (mg GAE/100 g)
A. oryzae			
0 hr	28.6 ± 0.8 c	76.3 ± 0.5 b	3.8 ± 0.2 a
2 hr	21.3 ± 0.5 b	73.5 ± 0.5 b	4.5 ± 0.1 b
4 hr	19.4 ± 0.1 a	60.2 ± 0.7 a	5.4 ± 0.0 c
6 hr	19.9 ± 0.4 ab	59.7 ± 0.3 a	5.5 ± 0.0 c
A. niger			
0 hr	25.1 ± 0.4 c	80.2 ± 0.7 c	3.4 ± 0.1 a
2 hr	24.1 ± 0.7 bc	73.5 ± 0.4 b	3.6 ± 0.0 a
4 hr	22.7 ± 0.8 ab	62.9 ± 0.8 a	3.7 ± 0.0 a
6 hr	22.2 ± 0.6 a	61.9 ± 0.7 a	4.6 ± 0.3 b

Note: All measurements were performed in triplicate on the composite blend from three fermented batches. Data represent the mean ± one standard deviation ($n = 3$). Different superscript letters within a column for each fungus represent significantly different values ($p < .05$).
Abbreviations: PPEF, pea protein-enriched flour; TIA, trypsin inhibitor activity; CIA, chymotrypsin inhibitor activity; TPC, total phenolic content; *A. oryzae*, *Aspergillus oryzae*; *A. niger*, *Aspergillus niger*; GAE, gallic acid equivalent.

between fermentation time and fungal strains ($p < .001$) (Table 2). TIA decreased in fermented PPEF over the duration of fermentation by *A. oryzae* and *A. niger* from 28.6 to 19.9 TIU/mg and 25.1 to 22.2 TIU/mg, respectively (Table 1). CIA decreased over the 6 hr of fermentation time from 76.3 to 59.7 CIU/mg and 80.2 to 61.9 CIU/mg for *A. oryzae*- and *A. niger*-fermented PPEF samples, respectively (Table 1). During SSF, peptide bonds are hydrolyzed yielding free amino acids through fungal activity; thus, it is hypothesized that trypsin and chymotrypsin inhibitors were reduced through this cleavage of the peptide chain yielding smaller, more soluble proteins (Fawale, Gabdamosi, Ige, & Kadiri, 2017).

A reduction in TIA has been demonstrated in previous studies following the fermentation of *Vigna racemose* flour (by *Lactobacillus fermentum* and *A. niger*), soybean meal (by *Lactobacillus brevis* and *A. oryzae),* and *Phaseolus vulgaris* (via natural fermentation) (Baramapama & Simard, 1994; Difo, Onyike, Ameh, Njoku, & Ndidi, 2015; Gao, Wang, Zhu, & Qian, 2013; Granito et al., 2002). Additionally, indigenous-microbial fermentation has been demonstrated to decrease the TIA of flour and whole bean seeds (*Phaseolus vulgaris*) (Granito et al., 2002). Gao et al. (2013) found SSF to be an effective process to reduce TIA in soybean meal when inoculated with *L. brevis* or *A. oryzae,* showing a 57.1% and 89.2% reduction, respectively. In comparison, TIA of PPEF underwent a 30.4% and 11.5% reduction when inoculated with *A. oryzae* and *A. niger,* respectively.

CIA showed a similar trend to TIA and thus decreased over fermentation time in PPEF samples by 21.8% and 22.8% for *A. oryzae* and *A. niger*, respectively (Table 1). Both chymotrypsin and trypsin inhibitors are low molecular weight proteins, making them heat-labile. Thermal treatment affects the intermolecular bonds responsible for maintaining the inhibitor's tertiary structure, causing breaks and changes to the active site conformation (Aviles-Gaxiola, Chuck-Hernandez, & Serna Saldivar, 2018). Thermal treatments have been extensively studied and shown to degrade inhibitors in legumes. Specifically, in pea seeds, boiling (100°C for 20 min) and pressure cooking (120°C for 10 min) inactivated TIA

TABLE 2 Two-way analysis of variance (ANOVA) analysis of fermented PPEF bioactive properties and protein quality

	Bioactive properties			Protein quality	
	TIA	CIA	TPC	IVPD	IV-PDCAAS
(a) Main effects					
Fungi	$p < .001$	$p < .001$	$p < .001$	$p < .001$	NS
Time	$p < .001$	$p < .001$	$p < .001$	$p < .001$	$p < .001$
(b) Interactions					
Fungi × time	$p < .001$	$p < .001$	$p < .001$	$p < .001$	$p < .001$

Abbreviations: PPEF, pea protein-enriched flour; TIA, trypsin inhibitor activity; CIA, chymotrypsin inhibitor activity; TPC, total phenolic content; IVPD, in vitro protein digestibility; IV-PDCAAS, in vitro protein digestibility-corrected amino acid score; NS, not significant.

TABLE 3 Amino acid composition (g per 100 g of flour, on an *as-is* basis) for fermented PPEF

	A. oryzae				*A. niger*			
Amino acid	**FT = 0 hr**	**FT = 2 hr**	**FT = 4 hr**	**FT = 6 hr**	**FT = 0 hr**	**FT = 2 hr**	**FT = 4 hr**	**FT = 6 hr**
CP (%)	46.8	48.3	48.9	49.2	46.5	46.3	51.1	53.3
Moisture (%)	6.1	6.0	5.7	6.3	5.6	6.2	6.4	6.2
Aspartic Acid	4.61	4.97	4.64	4.89	4.60	4.55	4.45	4.50
Glutamic Acid	6.62	7.28	6.86	7.12	6.80	6.57	6.52	6.64
Serine	1.92	2.09	2.00	2.08	2.00	1.97	1.92	1.89
Glycine	1.39	1.59	1.52	1.55	1.45	1.40	1.41	1.45
Histidine[b]	0.86	0.96	0.90	0.91	0.88	0.84	0.79	0.85
Arginine	3.37	3.44	3.42	3.50	3.40	3.33	3.19	3.36
Threonine[b]	1.44	1.59	1.51	1.53	1.48	1.43	1.41	1.44
Alanine	1.44	1.59	1.49	1.56	1.46	1.43	1.40	1.44
Proline	1.62	1.79	1.73	1.77	1.66	1.64	1.61	1.62
Tyrosine	1.39	1.60	1.43	1.39	1.40	1.35	1.39	1.45
Valine[b]	1.42	1.64	1.55	1.46	1.46	1.37	1.37	1.60
Methionine[a,b]	0.44	0.53	0.44	0.45	0.45	0.45	0.46	0.46
Cysteine[a]	0.47	0.56	0.46	0.46	0.48	0.47	0.46	0.46
Isoleucine[b]	1.41	1.60	1.50	1.43	1.41	1.34	1.32	1.58
Leucine[b]	2.78	3.10	2.93	2.97	2.85	2.77	2.76	2.80
Phenylalanine[b]	1.95	2.20	2.08	2.09	1.98	1.91	1.91	1.99
Lysine[b]	2.83	2.96	2.74	2.96	2.72	2.77	2.60	2.81
Tryptophan[b]	0.45	0.46	0.45	0.43	0.45	0.45	0.42	0.44

Note: Measurements were performed once on each flour sample.

Abbreviations: PPEF, pea protein-enriched flour; *A. oryzae*, *Aspergillus oryzae*; *A. niger*, *Aspergillus niger*; FT, fermentation time; CP, crude protein, wet weight basis.

[a]Indicates sulfur amino acid.

[b]Indicates essential amino acids.

completely (Aviles-Gaxiola et al., 2018). In the current study, all of the samples were heated to 80°C to deactivate the fungi including the fermentation time 0 control sample; therefore, the reduction in TIA and CIA can be attributed to the fermentation process. However, since the 0-hr samples had significantly higher CIA and TIA compared with SSF-treated samples (2, 4, and 6 hr), the observed reductions after SSF were not the results of heat treatments, but of SSF. Analyses were not conducted on a raw PPEF sample to determine the effect of the 80°C heat treatment. Overall, the observed declines in protease inhibitor activity in the current study are mainly attributed to the fungi's hydrolysis of the protein into simpler complexes.

Phenolic compounds are known to be health-beneficial due to their antioxidant activity. The interaction between fermentation time and fungal strain was found to have a significant effect on total phenolic content (TPC) ($p < .001$) (Table 2). TPC increased throughout the fermentation time course, ranging from 3.8 to 5.5 mg GAE/100 g and 3.4 to 4.7 mg GAE/100 g for *A. oryzae* and *A. niger* PPEF samples, respectively (Table 1). In the literature, fermentation has been reported to increase the content of bioactive phenolic compounds in legumes and furthermore enhance their antioxidant activity. For example, TPC was reported to increase in lentil and soybean through SSF processing (Fernandez-Orozco et al., 2007; Lin, Wei, & Chou, 2006; Torino et al., 2013). SSF of lentil with *Bacillus subtilis* increased the TPC of lentil from 24 mg GAE/g to 34–35 mg GAE/g after 48 or 96 h of fermentation (Torino et al., 2013). This late-phase fermentation increase could be due to lentil's different composition of proximate constituents (i.e., protein, carbohydrate, lipid, and ash) when compared with pea. Fernandez-Orozco et al. (2007) observed an increase in phenolic content after fermentation with *A. oryzae, Rhizopus oryzae,* and *B. subtilis* of soybean. This was attributed to the fermentation process hydrolyzing complexes of polyphenols into simpler ones. Additionally, Duenas, Fernandez, Hernandez, Estrella, and Munoz (2005) stated that the complex polyphenols hydrolyzed during SSF resulted in simpler and superior biologically active compounds. Schmidt, Goncalves, Prietto, Hackbart, and Furlong (2014) reported an increase in free phenolic content through SSF by filamentous fungi's production of enzymes and attributed that result to the cleavage of

TABLE 4 Essential amino acid concentration (mg/g protein) and amino acid scores for fermented PPEF

	Amino acids								
	THR	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
(a) Essential amino acid concentration (mg/g protein)									
A. oryzae									
0 hr	31	30	19	30	59	71	18	61	10
2 hr	32	32	19	31	59	73	19	56	9
4 hr	31	32	19	31	60	72	18	56	9
6 hr	31	30	18	29	60	71	18	60	9
A. niger									
0 hr	32	31	20	30	61	73	19	59	10
2 hr	31	30	20	29	60	71	18	60	10
4 hr	28	27	18	26	54	65	16	51	8
6 hr	27	30	17	30	52	64	16	53	8
FAO Reference	34	35	25	28	66	63	19	58	11
(b) Amino acid score									
A. oryzae									
0 hr	0.90	0.87	0.77[a]	1.08	0.90	1.13	0.96	1.04	0.88
2 hr	0.96	0.93	0.76[a]	1.12	0.89	1.16	1.01	0.97	0.83
4 hr	0.91	0.90	0.74[a]	1.10	0.91	1.14	0.97	0.97	0.83
6 hr	0.92	0.85	0.74[a]	1.04	0.92	1.12	0.08	1.04	0.80
A. niger									
0 hr	0.94	0.90	0.80[a]	1.08	0.93	1.15	0.99	1.01	0.88
2 hr	0.91	0.85	0.79[a]	1.03	0.91	1.12	0.96	1.03	0.88
4 hr	0.81	0.77	0.72[a]	0.92	0.82	1.03	0.82	0.88	0.75
6 hr	0.79	0.86	0.69[a]	1.06	0.79	1.02	0.84	0.91	0.76

Note: Measurements were performed once on each flour sample.

Abbreviations: THR, threonine; CYS, cysteine; VAL, valine; MET, methionine; ILE, isoleucine; LEU, leucine; TYR, tyrosine; PHE, phenylalanine; HIS, histidine; LYS, lysine; TRP, tryptophan; *A. oryzae*, *Aspergillus oryzae*; *A. niger*, *Aspergillus niger*; PPEF, pea protein-enhanced flour.

[a]Indicates the first-limiting amino acid.

compounds complexed with lignin. PPEF contains approximately ~45% of each carbohydrate and protein, and phenolic compounds are trapped within protein–carbohydrate matrix. Thus, the cleavage of carbohydrate and protein during SSF could increase the total phenolic contents, that is, liberation of phenolics. The increase in phenolic contents through fermentation has also been demonstrated to improve health-linked functionality due to conjugate forms of phenolic compounds becoming bioconverted into their free forms (Torino et al., 2013). Therefore, the overall quality of PPEF can be improved if the phenolic complexes are hydrolyzed into their free conjugate forms during SSF. It should be noted though that fermented plant protein is a complex material containing peptides and amino acids released during fermentation that can interfere with the total phenolic assay and as such gives no indication of the specific changes taking place within the phenolic compounds as a result of fermentation.

3.2 | Protein quality

Protein quality is an important criterion for adequate nutrition and maintenance of good health. A major drawback of plant pulse crops such as peas is their low digestibility. The digestibility of pulse protein could be greatly influenced by the globular structure and conformation of protein as well as the presence of bioactive compounds, for example, protease inhibitors. As shown above, SSF of PPEF reduced the activities of protease inhibitors, allowing improved protease activities. Thus, processing through fermentation of PPEF provides an opportunity to increase protein digestibility and amino acid availability. The full amino acid composition of fermented PPEF, reported in grams per 100 g of flour, is given in Table 3, with the essential amino acid content (mg EEA per g protein) and the amino acid score given in Table 4. The limiting amino acids of fermented PPEF throughout the fermentation time course remained methionine and cysteine (Table 5). The

limiting amino acid values in fermented PPEF ranged from 0.74 to 0.77 for *A. oryzae* and 0.69 to 0.80 for *A. niger* (Table 5). The limiting amino acid score for fermented PPEF is consistent with the published literature since legumes are known to be limiting in sulfur amino acids and tryptophan (GL-Pro, 2005; Nosworthy et al., 2018). The decrease over fermentation time could be due to the fungi utilizing these amino acids, further reducing the essential amino acids. Nosworthy et al. (2018) similarly determined methionine and cysteine to be the limiting amino acids in red and green lentils when processed through various methods such as extrusion, cooking, and baking. In their study, the lentils ranged from 0.57 for baked green lentil to 0.68 for extruded red lentils.

The in vitro protein digestibility (IVPD) of fermented PPEF increased over fermentation time from 74.8% to 80.9% and 74.9% to 79.4% for *A. oryzae* and *A. niger* samples, respectively (Table 5). The IVPD had a statistically significant interaction with fermentation time and fungal strains ($p < .001$) (Table 2). During SSF, the production of fungal proteases hydrolyzed proteins into smaller peptide chains and amino acids making them more easily accessible to digestive enzymes as well as cleaved trypsin and chymotrypsin inhibitors, which both in turn made the fermented PPEF more digestible over time.

Fermentation was reported in previous studies to significantly improve the IVPD for cereal and legume samples. Chandra-Hioe, Wong, and Arcot (2016) found an increase in IVPD with fermentation for desi chickpea but not for kabuli chickpea or faba bean. This effect was assumed to be due to the increase in proteolytic activity in the fermented desi chickpea as the TIA did not significantly decrease with fermentation. Angulo-Bejarano et al. (2008) found IVPD of chickpea flour was improved 15% by SSF; proteins in the tempeh flour had an IVPD of 83.20%. The authors attributed this to the elimination of antinutritional factors through hydrolytic reactions and the heating step of 90°C for 30 min causing protein denaturation. Furthermore, other studies showing increases in IVPD of fermented products were presumably due to the opening of proteins during SSF through protease activity resulting in a partially digested protein (Alka, Neelam, & Shruti, 2012; Fawale et al., 2017; Granito et al., 2002; Yousif & Tinay, 2001). The increase in IVPD in this study would be a result of TIA decrease through SSF (Table 1). It is also hypothesized that the decrease in pH caused by fermentation would heighten endogenous protease activity resulting in more readily digestible polypeptides through the hydrolysis of larger proteins (Alka et al., 2012).

In vitro protein digestibility amino acid score (IV-PDCAAS) was also used to determine the protein quality, a method that takes amino acid score into account. The reduction in bioactive compounds and the alteration of amino acid profile of a protein source through fermentation would alter the IV-PDCAAS. PDCAAS is the main test used by the Food and Agriculture Organization of the United Nations (FAO) for assessing global food protein quality since it incorporates essential amino acids of food protein and digestibility is an accurate analysis. Complete proteins have PDCAAS of 100%. IV-PDCAAS of PPEF decreased over the fermentation time from 66.7% to 63.5% and 69.3% to 59.0% for *A. oryzae* and *A. niger*, respectively (Table 5). The IV-PDCAAS had a statistically significant interaction with fermentation time and fungal strain on fermented PPEF ($p < .001$) (Table 2). The reduction in IV-PDCAASs in the fermented PPEF reflects the reduction in the limiting amino acid. IV-PDCAAS

TABLE 5 Amino acid scores and protein data of fermented PPEF

Sample	Limiting amino acid[a]	Limiting amino acid score[a]	IVPD[b] (%)	IV-PDCAAS[b] (%)
A. oryzae				
0 hr	MET + CYS	0.77	74.8 ± 0.5	66.7 ± 0.3
2 hr	MET + CYS	0.76	71.1 ± 0.7	65.0 ± 0.1
4 hr	MET + CYS	0.74	79.8 ± 0.6	63.3 ± 0.3
6 hr	MET + CYS	0.74	80.9 ± 0.4	63.5 ± 0.0
A. niger				
0 hr	MET + CYS	0.80	74.9 ± 0.8	69.3 ± 0.1
2 hr	MET + CYS	0.79	74.2 ± 0.4	68.7 ± 0.2
4 hr	MET + CYS	0.72	75.4 ± 0.3	61.1 ± 0.1
6 hr	MET + CYS	0.69	79.4 ± 0.9	59.0 ± 0.1

Abbreviations: MET, methionine; CYS, cysteine; IVPD, in vitro protein digestibility; IV-PDCAAS, in vitro protein digestibility-corrected amino acid score; *A. oryzae*, *Aspergillus oryzae*; *A. niger, Aspergillus niger*; PPEF, pea protein-enriched flour.

[a]Measurements were performed once on one composite sample from the triplicate batches.

[b]Measurements were performed in triplicate on the composite blend from three batches of fermentation. Data represent the mean ± one standard deviation. Different superscript letters within IVPD for each fungus represent significantly different values ($p < .05$).

of processed yellow pea has been reported as 62.27% using extrusion and 67.4% using cooking indicating that processing method strongly influenced protein quality (Nosworthy et al., 2017). Fermentation was an effective processing method to increase digestibility; however, limiting amino acids were decreased (via fungi utilization) which in turn decreased the IV-PDCAAS by 4.8% for *A. oryzae-fermented* and 14.9% for *A. niger*-fermented samples.

4 | CONCLUSION

Overall, trypsin and chymotrypsin inhibitor activities decreased over the duration of fermentation, whereas total phenolic content increased. This occurred due to the production of hydrolytic enzymes during fermentation; in the case of trypsin and chymotrypsin, they became hydrolyzed and inactivated. The IVPD increased with fermentation, whereas the IV-PDCAAS decreased, due to the decrease in the limiting amino acids methionine and cysteine, resulting from fermentation. Since SSF with *A. niger* and *A. oryzae* led to decreased protein quality, it is not recommended as a means for altering the nutritional value of pea protein-enriched flour. However, fermentation with other GRAS microorganisms may lead to differing outcomes.

ACKNOWLEDGMENTS

Financial support for this research was provided by the Global Institute for Food Security (Saskatoon, SK, Canada) and the Saskatchewan Agricultural Development Fund (# 20140283).

ORCID

Hayley M. Kumitch https://orcid.org/0000-0003-3509-2788
Michael T. Nickerson https://orcid.org/0000-0002-9040-5639
Takuji Tanaka https://orcid.org/0000-0003-3304-5830

REFERENCES

Alka, S., Neelam, Y., & Shruti, S. (2012). Effect of fermentation on physicochemical properties & *in vitro* starch and protein digestibility of selected cereals. *International Journal of Agriculture and Food Science*, *2*(3), 66–70.

Angulo-Bejarano, P. I., Verdugo-Montoya, N. M., Cuevas-Rodriguez, E. O., Milan-Carrillo, J., Mora-Escobedo, R., Lopez-Valenzuela, J. A., … Reyes-Moreno, C. (2008). Tempeh flour from chickpea (*Cicer arietinum* L.) nutritional and physicochemical properties. *Food Chemistry*, *106*(1), 106–112.

Association of Official Analytical Chemists (AOAC) (2005). Methods 22–40.01, 984.13A, 925.10, 982.30, 985.28. Official methods of analysis of association official analytical chemists. Journal of AOAC International.

Astephen, N. (2018). Waters AccQ-Tag method for hydrolysate amino acid analysis. Retrieved from http://www.waters.com/waters/library.htm?cxml:id=511436&lxml:id=1520760&locale=120

Aviles-Gaxiola, S., Chuck-Hernandez, C., & Serna Saldivar, S. O. (2018). Inactivation methods of trypsin inhibitor in legumes: A review. *Journal of Food Science*, *83*(1), 17–29. https://doi.org/10.1111/1750-3841.13985

Bai, T., Nosworthy, M. G., House, J. D., & Nickerson, M. T. (2018). Effect of tempering moisture and infrared heating temperature on the nutritional properties of desi chickpea and hull-less barley flours, and their blends. *Food Research International*, *108*(1), 430–439. https://doi.org/10.1016/j.foodres.2018.02.061

Barampama, Z., & Simard, R. E. (1994). Oligosaccharides, antinutritional factors, and digestibility of dry beans as affected by processing. *Journal of Food Science*, *59*(4), 833–838.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, *43*(2), 414–431. https://doi.org/10.1016/j.foodres.2009.09.003

Çabuk, B., Nosworthy, M. G., Stone, A. K., Korber, D. R., Tanaka, T., House, J. D., & Nickerson, M. T. (2018). Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technology and Biotechnology*, *56*(2), 257–264. https://doi.org/10.17113/ftb.56.02.18.5450

Chandra-Hioe, M. V., Wong, C. H., & Arcot, J. (2016). The potential use of fermented chickpea and faba bean flour as food ingredients. *Plant Foods for Human Nutrition*, *71*(1), 90–95. https://doi.org/10.1007/s11130-016-0532-y

Cuevas-Rodriguez, E., Milan-Carrillo, J., Mora-Escobedo, R., Cardenas-Valenzuela, O., & Reyes-Moreno, C. (2004). Quality protein maize (*Zea mays* L.) tempeh flour through solid state fermentation process. *LWT – Food Science and Technology*, *37*(1), 59–67.

Deshpande, S., Salunkhe, D., Oyewole, O., Azam-Ali, S., Battcock, M., & Bressani, R. (2000). *Fermented grain legumes, seeds and nuts: a global perspective*, Vol. *142*. Rome: Food & Agriculture Organization of the United Nations.

Difo, V. H., Onyike, E., Ameh, D. A., Njoku, G. C., & Ndidi, U. S. (2015). Changes in nutrient and antinutrient composition of *Vigna racemosa* flour in open and controlled fermentation. *Journal of Food Science Technology*, *52*(9), 6043–6048.

Duenas, M., Fernandez, D., Hernandez, T., Estrella, I., & Munoz, R. (2005). Bioactive phenolic compounds of cowpeas (*Vigna sinensis* L.) modifications by fermentation with natural microflora and with *Lactobacillus plantarum* ATCC 14917. *Journal of the Science of Food and Agriculture*, *85*(1), 297–304.

FAO (1991). *The state of food and agriculture*. Retrieved from http://www.fao.org/3/a-t0496e.pdf

Fawale, O. S., Gabdamosi, S. O., Ige, M. M., & Kadiri, O. (2017). Effects of cooking and fermentation on the chemical composition, functional and antinutritional properties of kariya (*Hildergardia barteri*) seeds. *Food Science & Nutrition*, *5*(1), 1106–1115.

Fernandez-Orozco, R., Frias, J., Munoz, R., Zielinski, H., Piskula, M. K., Kozlowska, H., & Vidal-Valverde, C. (2007). Fermentation as a Bio-process to obtain functional soybean flours. *Journal of Agricultural and Food Chemistry*, *55*(1), 8972–8979.

Gao, Y. I., Wang, C. S., Zhu, Q. H., & Qian, G. Y. (2013). Optimization of solid-state fermentation with *Lactobacillus brevis* and *Aspergillus oryzae* for trypsin inhibitor degradation in soybean meal. *Journal of Integrative Agriculture*, *12*(5), 869–876.

GL-Pro (2005). *Guidelines for growing grain legumes in Europe*. Retrieved from http://www.grainlegumes.com/aep/production/growing_gl_in_europe/guidelinesforgrowing_gl_in_europe

Granito, M., Frias, J., Doblado, R., Guerra, M., Champ, M., & Vidal-Valverde, C. (2002). Nutritional improvement of beans (*Phaseolus vulgaris*) by natural fermentation. *Europe Food Research Technology*, *214*(1), 226–231.

International Organization for Standardization (2016) Animal feeding stuffs – Determination of tryptophan content ISO 13904. Geneva.

Kannan, S., Nielsen, S. S., & Mason, A. C. (2001). Protein digestibility-corrected amino acid scores for bean and bean-rice infant weaning food products. *Journal of Agricultural & Food Chemistry*, *49*, 5070–5074. https://doi.org/10.1021/jf010323u

Kumitch, H. M. (2019). *The effect of solid-state fermentation on air-classified pea protein-enriched flour to improve the digestibility and functional properties (Master's thesis)*. Saskatoon, Canada: University of Saskatchewan.

Lin, C., Wei, Y., & Chou, C. (2006). Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. *Food Microbiology*, *23*(1), 628–633. https://doi.org/10.1016/j.fm.2005.12.004

Makkar, H. P. S., Siddhuraju, P., & Becker, K. (2007). *Plant secondary metabolites*. Totowa, New Jersey: Humana Press.

Meinlschmidt, P., Ueberham, E., Lehmann, J., Schweiggert-Weisz, U., & Eisner, P. (2005). Immunoreactivity, sensory and physicochemical properties of fermented soy protein isolate. *Food Chemistry*, *205*(1), 229–238.

Millward, D. J., Layman, D. K., Tome, D., & Schaafsma, G. (2008). Protein quality assessment: Impact of expanding understanding of protein and amino acid needs for optimal health. *American Journal of Clinical Nutrition*, *87*(5), 1576S–1581S. https://doi.org/10.1093/ajcn/87.5.1576S

Nosworthy, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., … House, J. D. (2017). Effect of processing on the *in vitro* and *in vivo* protein quality of yellow and green split peas (*Pisum sativum*). *Journal of Agricultural and Food Chemistry*, *65*(1), 7790–7796.

Nosworthy, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., … House, J. D. (2018). Effect of processing on the *in vitro* and *in vivo* protein quality of red and green lentils (*Lens culinaris*). *Food Chemistry*, *240*(1), 588–593. https://doi.org/10.1016/j.foodchem.2017.07.129

Pelgrom, P., Vissers, A., Boom, R., & Schutyser, M. (2013). Dry fractionation for the production of functional pea protein concentrates. *Food Research International*, *53*, 232–239.

Sandberg, A. S. (2002). Bioavailability of minerals in legumes. *British Journal of Nutrition*, *88*(3), 281–285. https://doi.org/10.1079/BJN/2002718

Schmidt, C. G., Goncalves, L. M., Prietto, L., Hackbart, H. S., & Furlong, E. B. (2014). Antioxidant activity and enzyme inhibition of phenolic acids from fermented rice bran with fungus *Rhizopus oryzae*. *Food Chemistry*, *146*(1), 371–377.

Schutyser, M. A. I., Pelgrom, P. J. M., van der Goot, A. J., & Boom, R. M. (2015). Dry fractionation for sustainable production of functional legume protein concentrates. *Trends in Food Science & Technology*, *45*(2), 327–335. https://doi.org/10.1016/j.tifs.2015.04.013

Shi, L., Mu, K., Arntfield, S. D., & Nickerson, M. T. (2017). Changes in levels of enzyme inhibitors during soaking and cooking for pulses available in Canada. *Journal of Food Science Technology*, *54*(4), 1014–1022. https://doi.org/10.1007/s13197-017-2519-6

Torino, M. I., Limon, R. I., Marinez-Villaluenga, C., Makinen, S., Pihlanto, A., Vidal-Valverde, C., & Frias, J. (2013). Antioxidant and antihypertensive properties of liquid and solid state fermented lentils. *Food Chemistry*, *136*(2), 1030–1037. https://doi.org/10.1016/j.foodchem.2012.09.015

Waterman, P. G., & Mole, S. (1994). *Analysis of phenolic plant metabolites*. Oxford: Blackwell Scientific Publications.

Yousif, N. E., & Tinay, A. H. (2001). Effect of fermentation on sorghum protein fractions and in vitro protein digestibility. *Plant Foods for Human Nutrition*, *56*(1), 175–182.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Kumitch HM, Stone A, Nosworthy MG, et al. Effect of fermentation time on the nutritional properties of pea protein-enriched flour fermented by *Aspergillus oryzae* and *Aspergillus niger*. *Cereal Chem*. 2019;97:104–113. https://doi.org/10.1002/cche.10234