Aspergillus niger Prolyl Endoprotease (AN-PEP), a Unique Enzyme that Breaks Down Gluten

Research Review

BACKGROUND

Gluten is a dietary protein found in foods made from wheat, barley, and rye, which are dietary mainstays for many people. Other grain products like oats may also contain small amounts of gluten that are picked up during processing.[1] There are also many other potential sources of gluten (**Table 1**).

Gluten is a protein composite and is made up gliadins and glutenins in roughly equal parts.[2] Gliadins and glutenins are made up of several subtypes (**Figure 1**). Unfortunately, the gluten proteins may be difficult for the digestive enzymes in the human gastrointestinal (GI) tract to break down due to being unusually high in the amino acid proline; this is true even in healthy individuals.[2-4] Many patients and consumers avoid wheat and gluten due to a spectrum of health issues—including allergy to wheat and sensitivity or intolerance to gluten, as well as celiac disease (CD).[5]

CD is an autoimmune disorder that is triggered by an inappropriate T cell-mediated immune response to dietary gluten, particularly to α-gliadins.[4,6] The pathogenesis of CD results in the malabsorption of nutrients and chronic inflammation of the small intestine mucosa on both a macroscopic and microscopic level.[6,7] CD symptoms are typically GI-related, but extraintestinal signs and symptoms may manifest in many other organ systems. Atypical presentations of CD may be neurologic, musculoskeletal,

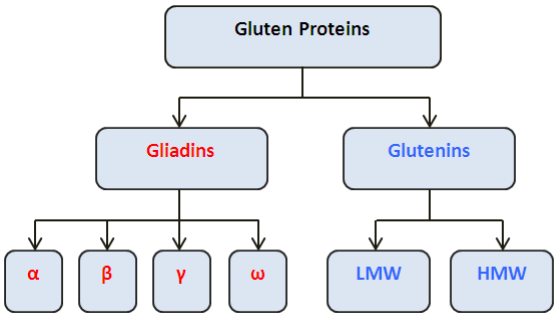

Figure 1. Gluten is a protein complex made up of several gliadin and glutenin subtypes. (Abbreviations: α = alpha; β = beta; γ = gamma; ω = omega; LMW = low molecular weight; HMW = high molecular weight.)

psychiatric, dermatologic, hematologic, and/or endocrine-related (including affecting fertility).[7,8] Epidemiologic data indicates that CD prevalence has been doubling about every 20 years, and its current prevalence is 1% in the general population. The vast majority of those with CD, however, have *not* been diagnosed or treated.[9-11]

A cornerstone of treatment for CD is the avoidance of dietary gluten.[12] However, a systematic review of 38 studies in adults with CD showed that strict adherence to a gluten-free diet ranged from 42% to 91%.[13] Furthermore, 50% of patients with CD who attempt to strictly adhere to a gluten-free diet do not experience symptom resolution.[14] In this population, known as having non-responsive celiac disease (NRCD), the most common etiology is gluten exposure and contamination—despite attempts at eating a gluten-free diet.[15-17]

Gluten may also trigger symptoms in those with non-celiac gluten sensitivity (NCGS). Symptoms are similar to CD, but with an emphasis on extraintestinal symptoms—such as behavioral changes, bone or joint pain, muscle cramps, leg numbness, weight loss, and chronic fatigue.[5]

Table 1. **Sources of Gluten Are Ubiquitous**

Sources of Gluten	Common Foods that Contain Gluten	Foods that May Contain Gluten	Other Items that May Contain Gluten
Wheat, as well as varieties and derivatives of wheat: - wheatberries - durum - emmer - semolina - spelt - farina - farro - graham - khorasan wheat - einkorn wheat Wheat starch Rye Barley Triticale (wheat/rye hybrid)	Pastas Noodles Breads and pastries Crackers Baked goods Cereal and granola Breakfast foods - pancakes, waffles, biscuits Breading and coating mixes Croutons Sauces and gravies - many use flours as thickeners - soy sauce - cream sauces made with a roux Flour tortillas Malt in various forms Beer Brewer's yeast	Energy bars/granola bars French fries (battered) Potato chips (seasoned) Processed lunch meats Candy, candy bars, and mints Soups - flour may be used as a thickener - may contain barley Multi-grain tortilla chips or tortillas Salad dressings and marinades Starch or dextrin Brown rice syrup Meat substitutes - seitan, vegetarian burgers, etc. Soy sauce	Lipstick and other cosmetics Lip gloss and lip balm Body care products Communion wafers Vitamins Herbal or nutritional supplements Drugs Over-the-counter medications Art and craft supplies - modeling clay - glue

Adapted from the Celiac Disease Foundation website.[18]

NCGS prevalence has been difficult to establish, in part because it has only recently been described in medical literature.[5] Prevalence is estimated to be as low as 0.55%.[19,20] However, population-based studies in Northern Europe have described the prevalence of irritable bowel syndrome to be 16% to 25%, with coincident NCGS estimated to be 28%.[21] Thus, the actual prevalence may be much higher. Those with NCGS may also struggle to maintain a gluten-free diet.

Adherence to a gluten-free diet is difficult for all ages, but especially for teens and college students.[7,22] Avoiding gluten is often difficult due to the abundance of gluten in dietary staples like cereals, breads, and other baked goods. Those avoiding wheat and gluten may also inadvertently eat less obvious sources of gluten (e.g., soups thickened with wheat flour.)

ENZYMES THAT BREAK DOWN GLUTEN

Oral supplementary digestive enzymes represent a promising adjunctive strategy for individuals avoiding dietary gluten.[23,24]

AN-PEP

Aspergillus niger prolyl endoprotease (AN-PEP) is a unique enzyme that breaks down gluten. It is naturally occurring and has been isolated from *A. niger,* a microorganism commonly cultured to make supplement-grade ingredients, including other commonly-used digestive enzymes and citric acid. AN-PEP is an "endoprotease"—meaning that it breaks down protein over the *entire* length of protein and peptide chains, rather than only on the N- and C-terminal ends.[25] Many naturally occurring enzymes in the human GI tract are also endoproteases, including pepsin, trypsin, and chymotrypsin. AN-PEP has been assessed in vitro, as well as in several clinical studies.

AN-PEP Is Resistant to the Stomach's Acidic Conditions *and* Remains Active in the GI Tract

Enzymes capable of breaking down protein must be resistant to highly acidic conditions in the stomach. In vitro data have shown that AN-PEP functions optimally in acidic conditions:

- The enzymatic activity of AN-PEP was measured over a broad pH range and had a pH optimum of 4.5 (**Figure 2**).[25,26]

Enzymatic digestive aids must also be able to resist being degraded by endoprotoease enzymes that are naturally occurring in the GI tract. In vitro data have shown that AN-PEP retains enzymatic function in the presence of endogenous digestive enzymes over a broad pH range:

- The enzymatic activity of AN-PEP was measured at pH 2 in the presence of pepsin, an enzyme produced by the chief cells in the stomach. AN-PEP retained nearly 100% of its enzymatic activity for 1 hour (total time tested; **Figure 3**).[26]

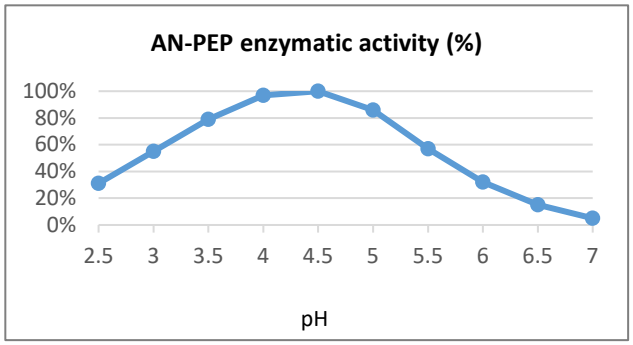

Figure 2. AN-PEP is active over a broad pH range and has an acidic pH optimum. (Data courtesy of DSM Food Specialties, Delft, The Netherlands.)

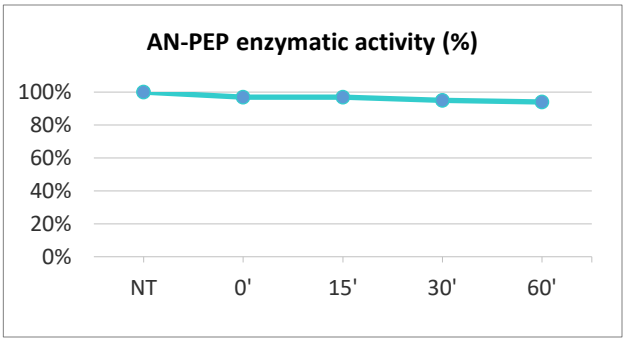

Figure 3. AN-PEP is resistant to highly acidic pH and digestion by the endogenous enzyme pepsin. AN-PEP was incubated at pH 2.0 with pepsin. At each time point, the reaction was stopped, and the activity of AN-PEP was measured at its pH optimum. (NT = not treated.) (Data courtesy of DSM Food Specialties, Delft, The Netherlands.)

AN-PEP Breaks Down Intact Gluten Proteins

In vitro research has shown that AN-PEP breaks down whole gluten proteins:

- AN-PEP and a gluten solution were combined for 2 hours. The mixture was then separated via HPLC and analyzed by mass spectrometry. AN-PEP broke down intact gluten into 152 unique peptides. (Dosage: 5 mg AN-PEP per gram of gluten.)[26]

AN-PEP Breaks Down Gluten Peptides *and* Cleaves Them after the Amino Acid Proline

In order to effectively prevent the stimulation of T cells, enzymes need to break down gluten epitopes (fragments) that are known to trigger T cell-mediated immune responses. In vitro assays have demonstrated that AN-PEP cleaved every T cell-stimulatory epitope tested, including α-gliadin peptide AA 31-49 (Glia 31-49), a gluten epitope considered toxic to those with CD.[27-29] AN-PEP was incubated with 13 different gluten peptides that are known to stimulate T cells. Mass spectrometry confirmed that AN-PEP broke down each of the 13 peptides. In addition, it was shown that AN-PEP cleaved the peptides specifically after the amino acid proline (**Table 2**).[26]

Gluten Peptide	Major Cleavage Sites
Glia 31–49	LGQQQP✂FPPQQP✂YP✂QPQPF
Glia-α-2	P✂QPQLP✂YPQPQLPY
Glia-α-9	QLQP✂FP✂QPQLP✂Y
Glia-α-20	PFRP✂QQP✂YP✂QPQPQ
Glia-γ-1	QPQQP✂QQSFP✂QQQRP✂F
Glia-γ-2	QQP✂YPQQP✂QQPFPQ
Glia-γ-30	VQGQGIIQP✂QQPAQL
Glt-17	QQPP✂FSQQQQQP✂LPQ
Glt-156	QQPP✂FSQQQQSP✂FSQ
Glu-5	QQUSQP✂QUP✂QQQQUP✂QQPQQF
Glu-21	QPQP✂FP✂QQSEQSQQP✂FQPQPF
DQ8-Glt	QQGYYP✂TSP✂QQS
DQ8-Glia	SGQGSFQP✂SQQN

Table 2. Gluten peptides known to stimulate T cells were treated with AN-PEP at pH 4.5. Resultant peptide fragments were identified by MALDI-TOF-MS. Cleavage sites indicated by ✂.

Gluten Peptide	Half-Life (minutes)
α-Gliadin (Glia-α-9)	3.87
γ-Gliadin (Glia-γ-1)	2.36
LMW-Glutenin (Glt-156)	5.80
HMW-Glutenin	6.19

Table 3. AN-PEP rapidly breaks down gluten peptides under acidic conditions.

AN-PEP Breaks Down Gluten Peptides Rapidly

Under ideal circumstances, gluten should be efficiently broken down in the stomach to prevent gluten peptides from triggering the stimulation of T cells in the small intestine. In vitro research has shown that AN-PEP cleaves gluten peptides rapidly:

- To test gluten protein breakdown speed, AN-PEP was incubated with 4 different gluten peptides (α-gliadin, γ-gliadin, LMW-glutenin, and HMW-glutenin) at pH 4.5. Rapidly breaking down the gluten peptides, the half-life of the reactions ranged from 2.4 to 6.2 minutes (**Table 3**).[26]
- In another study, breakdown of gliadin peptides by AN-PEP under acidic conditions was visible via Western blot within 30 minutes at dosages as low as 33,000 PPI (protease picomol international) AN-PEP per gram of gluten.[30]

AN-PEP Prevents T Cell Stimulation by Gluten Peptides

Part of the pathophysiology of gluten-related autoimmune disease involves the stimulation of T cells by gluten. In vitro research has shown that AN-PEP can prevent this stimulation:

- AN-PEP was incubated with gluten along with pepsin, trypsin, and chymotrypsin. At several time points, an antibody-based assay was used to detect 4 different gluten epitopes that are known to stimulate T cells. After 30 minutes, 2 gluten epitopes (α-gliadin, γ-gliadin) could no longer be detected. After 90 additional minutes, another gluten epitope (LMW-glutenin) could no longer be detected, and 90% of a fourth gluten epitope (HWM-glutenin) had been destroyed. (Dosage: 1 mg AN-PEP per gram of gluten.)[26]
- The ability of AN-PEP to digest a gluten-containing meal and subsequently prevent T cell stimulation was tested using a TIM system, an artificial digestive tract model. Compared to the control, AN-PEP accelerated the breakdown of gliadins and glutenins in each of the 4 model compartments (corresponding to the stomach, duodenum, jejunum, and ileum) as detected by Western blot. Compared to the control, the gluten-containing meal with AN-PEP negated T cell stimulatory activity/detection of 4 tested gluten epitopes. (Dosage: 200 mg AN-PEP per gram of gluten.)[31]

AN-PEP in Clinical Studies

The ability of AN-PEP to digest gluten has been tested and verified in human volunteers: [32]

- In a randomized, double-blind, placebo-controlled, crossover study, 12 healthy volunteers were given gluten-containing meals on 4 separate test days in random order: low-calorie meal (143 kCal) with AN-PEP, low-calorie meal with placebo, high-calorie meal (405 kCal) with AN-PEP, or high-calorie meal with placebo. Despite caloric content variation, all meals contained a fixed amount of gluten protein (4 grams).
- Meals were administered through a nasoduodenal catheter with a triple lumen design that allowed collection of gastric and duodenal aspirates, which were then analyzed by ELISA and Western blot to determine breakdown of α-gliadin (**Figure 4**).
- Compared to placebo, AN-PEP significantly lowered α-gliadin concentration in the stomach and duodenum ($p < 0.001$) in low- and high-calorie meals. Within 1 hour, nearly all α-gliadin was degraded in the stomach. Furthermore, α-gliadin was nearly undetectable in the duodenal samples.
- The healthy volunteers were also monitored for GI symptoms during the study and it was found that there was no difference between AN-PEP and placebo. (Dosage: 333,320 PPI AN-PEP per gram of gluten.)

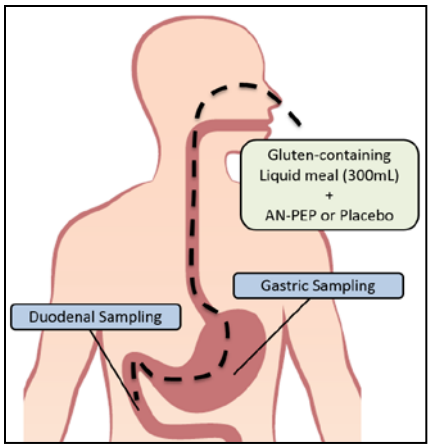

Figure 4. Gastric and duodenal samples were obtained via a nasoduodenal catheter then analyzed by ELISA and Western blot.

The safety and tolerability of AN-PEP has also been tested in adults with CD:

- In a randomized, double-blind, placebo-controlled study, CD subjects were asked to eat bread daily with either AN-PEP or placebo. The quality of life scores between the groups were similar, indicating that the AN-PEP was well-tolerated. (Dosage: 383,000 PPI AN-PEP per gram of gluten.)[33]

DPP-IV

Dipeptidyl peptidase IV (DPP-IV) is one of the most common enzymes in dietary supplements that support gluten digestion. Similar to AN-PEP, DPP-IV is naturally occurring and produced by a species of *Aspergillus*, as well as cleaves proteins/peptides after the amino acid proline. However, unlike AN-PEP, DPP-IV is an "exoprotease" and is so specific that it *only* cleaves peptides that have a proline in the second-to-last (penultimate) position from the N-terminus of a protein or peptide.[34] Due to this high degree of specificity, DPP-IV has been shown to be *unable* to break down gliadin peptides unless additional proteases are present.[4] In contrast, AN-PEP is able to digest gliadin peptides and gluten without requiring additional enzymes.[26,30-32] DPP-IV also loses its ability to break down gluten at pH <4.0—unlike AN-PEP, which functions at pH as low as 2.0.[25,26,34]

SUMMARY

Oral digestive enzymes represent a promising adjunctive treatment for those avoiding dietary gluten. AN-PEP is an extensively studied gluten-digesting enzyme that is naturally occurring. It is resistant to the harsh conditions of the GI tract, including its pH extremes and endogenous digestive enzymes.

AN-PEP rapidly breaks down gluten peptides and intact gluten proteins, helps prevent gluten peptides from stimulating T cells, and has been verified to safely break down gluten in clinical studies. The growing body of research on AN-PEP suggests that it is superior to the most commonly utilized digestive enzyme that was previously available. It has been shown to break down gluten in dosages as low as 33,000 PPI enzyme per gram of gluten in vitro and at dosages as low as 333,000 PPI enzyme per gram of gluten in clinical studies.

While it should not be used as a replacement for a gluten-free diet or as a treatment for celiac disease, AN-PEP could benefit patients who avoid dietary gluten. It may be also recommended as protection from inadvertent exposures to dietary gluten.

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