Glutenase and collagenase activities of wheat cysteine protease Triticain-α: Feasibility for enzymatic therapy assays


Abstract

Insufficient and/or improper protein degradation is associated with the development of various human pathologies. Enzymatic therapy with proteolytic enzymes aimed to improve insufficient proteolytic activity was suggested as a treatment of protease deficiency-induced disorders. Since in many cases human degradome is incapable of degrading the entire target protein(s), other organisms can be used as a source of proteases exhibiting activities distinct from human enzymes, and plants are perspective candidates for this source. In this study recombinant wheat cysteine protease Triticain-α was shown to refold in vitro into an autocatalytically activated proteolytic enzyme possessing glutenase and collagenase activities at acidic (or close to neutral) pH levels at the temperature of human body. Mass-spectrometry analysis of the products of Triticain-α-catalyzed gluten hydrolysis revealed multiple cleavage sites within the sequences of gliadin toxic peptides, in particular, in the major toxic 33-mer α-gliadin-derived peptide initiating inflammatory responses to gluten in celiac disease (CD) patients. Triticain-α was found to be relatively stable in the conditions simulating stomach environment. We conclude that Triticain-α can be exploited as a basic compound for development of (i) pharmaceuticals for oral administration aimed at release of the active enzyme into the gastric lumen for CD treatment, and (ii) topically active pharmaceuticals for wound debridement applications.

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1. Introduction

Protein expression followed by protein degradation is a basic feature of any organism. Both processes involve special mechanisms recognizing current requirements for protein composition and consequently inducing executive machinery for protein synthesis, translational repression or proteolytic digestion. While the induction or repression of protein synthesis eventually results in protein translation alterations, the proteases are the main enzymes responsible for protein degradation.

To accomplish protein degradation function, human degradome (the complete repertoire of proteases that are produced by the organism) consists of 570 proteases (Puente et al., 2003; Quesada et al., 2009), which, based on their catalytic mechanism, can be divided into five distinct classes: aspartic, metallo, cysteine, serine and threonine proteases (Rawlings et al., 2014). Human proteases also vary in substrate specificity, activity features, organ, tissue and cell type specific expression, and inter- and intracellular localization. As a result, the necessary degradation of endogenous human proteins as well as a number of exogenous proteins consumed as dietary components is achieved.

Abbreviations: BSA, bovine serum albumin; CD, celiac disease also known as Celiac Sprue; FDA, Food and Drug Administration; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Triticain-α-GM, wheat Triticain-α lacking signal peptide sequence and region for granulin domain.

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More than one hundred human hereditary diseases have been identified as a result of mutations within protease genes, most of which are recessive loss-of-function mutations (Puente et al., 2003; Quesada et al., 2009). Thus, the functions of at least of some human proteases are unique and cannot be complemented by the remaining subtypes of the proteases. Furthermore, some of the protein substrates are resistant to proteolysis by any of degradome components resulting in the development of pathological processes. For instance, insufficient cleavage of endogenous collagen fibrils results in fibrosis within different tissues (See et al., 2005; Rockey, 2006; Jinnin, 2010). Accumulation of aggregates of endogenous proteins and their cleavage products (e.g., amyloid beta (AB) peptides) are the hallmarks of prion encephalopathies, Alzheimer disease (AD), and Huntington disease (HD) (Soto, 2001; Puente et al., 2003; Aliev et al., 2014; Kosenko et al., 2014). A striking example of the disorder associated with incomplete exogenous proteins degradation is celiac disease (CD) also known as Celiac Sprue, an autoimmune disease strongly associated with the presence of human leukocyte antigen (HLA) class II alleles HLA-DQ2 and HLA-DQ8. The prevalence of CD is 1 in 100–300 individuals and it is primarily characterized by malabsorption of nutrients, affecting the intestines (Wieser and Koehler, 2008). The pathogenesis of CD involves inflammatory responses to exogenous gluten digestion products including a 33-mer Pro/Gln-rich α-gliadin-derived peptide (Shan et al., 2002).

A feasible approach for the treatment of CD and the other protease deficiency-induced pathologies is an enzymatic therapy with proteolytic enzymes aimed to supply insufficient proteolytic activity or to substitute for the defective enzyme (Puente et al., 2003; Bethune and Khosla, 2012; Wieser and Koehler, 2012). Efficacy of such therapy depends on properties of the protease(s) applied including their substrate specificity, stability and activity. Since in many cases human degradome is incapable of degrading the entire target protein(s), other organisms can be used as a source of proteases exhibiting activities distinct from human enzymes. Plants seem perspective candidates for this source. Each plant genome encodes more than 500 proteases but most of them remain uncharacterized (van der Hoorn, 2008; Pesquet, 2012). Meanwhile, it can be expected that they possess similar but mechanistically distinct activities as that of the human-encoded proteases. An illustrative example supporting this suggestion comes from the studies of the role of proteolytic enzymes in programmed cell death in human and plants (Sundström et al., 2009). It was found that one of the eukaryotic cell viability factors, an evolutionary conserved multifunctional protein Tudor-SN, is processed by caspase-3 during the apoptosis in human cells, whereas its plant close homologue is processed by metacaspase during the programmed cell death in spruce. In this case human caspase-3 and spruce metacaspase contribute to functionally similar processes by possessing different substrate specificities: caspase-3 is an Asp-specific protease (Crawford and Wells, 2011), whereas plant metacaspases possess Lys/Arg specificity (Vercammen et al., 2004; Bozhkov et al., 2005).

Remarkably, caspase-like activity is also found in plants but it is provided by serine protease Phytaspase, whereas human caspases belong to cysteine proteases family (Chichkova et al., 2010). Thus, the plant protein and its close homologue in human undergo different proteolytic processing depending on the nature of the corresponding enzymes. These data suggest that the plant degradome enzymes, along with hydrolysis of plant-specific proteins (van der Hoorn, 2008; Pesquet, 2012; Richau et al., 2012), may efficiently degrade protein targets in human organism and therefore may be considered as a perspective candidate for applications of enzymatic therapy aimed at complementing human protease dysfunctions. Furthermore, these enzymes might expand the potential of human proteolytic machinery in the treatment of pathologies developed as a result of dysfunction or insufficiency of human protein degradation pathways (Salas et al., 2008; Wieser and Koehler, 2012).

In the present work, we have studied the glutenase and collagene activity of the bacterially expressed wheat (Triticum aestivum) cysteine protease Triticain-α. Our results demonstrate feasibility of Triticain-α in the enzymatic therapy of CD aimed at detoxification of gluten at the stage of gastric digestion. Additional possible applications of Triticain-α as a therapeutic for wound debridement are also discussed.

2. Materials and methods

2.1. Construction of expression vectors

Total RNA was extracted from 3-days old germinating wheat plantlets with RNasy plant mini kit (Qiagen, USA) according to manufacturer’s protocol. Further molecular cloning was performed by the standard procedures (Sambrook et al., 1989). The fidelity of all obtained constructs was verified by sequencing. Triticain-α cDNA was obtained by reverse transcription using GGGGGATCCCTTACGGCTATTTCCTTGCAG oligonucleotide complementary to the 3’-region of Triticain-α mRNA gene (Gene Bank accession number AB267407). The obtained cDNA was amplified with the forward primer CC CATATGCATCATCATCATCATGATCCGAGAGGATCTAGTGCAG (Ndel restriction site is underlined), and the reverse primer GGG- GGATCTTTAGCAGCTATTTCCTGCAG (BamHI restriction site is underlined), and cloned into pET-42a(+) expression vector (Novagen, Germany) using Ndel and BamHI (Thermo Fisher Scientific Inc., USA) to construct expression vector for Triticain-α (pET-Triticain-α). To obtain an expression vector for the short-cut wheat Triticain-α (pQE-Triticain-α-GM) lacking signal peptide sequence and region for granulin domain (Fig. 1A), Triticain-α was amplified with the forward primer ATGGATCC-ATCGTGTCTGATCGGGAGAC (BamHI restriction site is underlined) and the reverse primer TATAAAGCTTATAAGCCCTTCTTGTCGG (HindII restriction site is underlined) using pET-Triticain-α as a template, and the obtained PCR product was subcloned into the pQE-80L expression vector (Qiagen, USA) using BamHI and HindIII (Thermo Fisher Scientific Inc., USA) restriction sites. The site-directed mutagenesis employing megaprimer method (Ke and Madison, 1997) was used to replace catalytic Cys of Triticain-α-GM to Ala. In particular, 141st TGC codon within Triticain-α-GM gene (154th codon within full-length Triticain-α) was substituted with GCT in two rounds of PCR using two external forward and reverse oligonucleotides, internal mutagenic megaprimer GCGGGAGCCTTGCGCTTTCACGC and pQE-Triticain-α-GM as a template. The final PCR product was subcloned into pQE80LB to obtain pQE-Triticain-α-GM154A.

2.2. Recombinant proteins expression and purification

BL21-CodonPlus(DE3)-RII or JM109 (Stratagene, USA) competent cells were transformed with pQE-Triticain-α-GM or pQE-Triticain-α-GM154A. Recombinant proteins expression was induced by 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Promega, USA). The protein expression levels were evaluated by SDS-PAGE. The biomass obtained from 200 mL of the culture medium was suspended in 5 mL of 0.01 M Tris–HCl buffer (pH 8.0). The cell suspension was sonicated using high-intensity ultrasonic processor (Cole Parmer, USA) (12 × 5 s, 0 °C) and centrifuged (10,000 × g, 15 min, 4 °C). The pellet was resuspended in 5 mL of the same buffer and centrifuged as described above. The washing procedure was repeated five times, after which 4 mL of 0.05 M Tris–HCl buffer (pH 8.0) containing 6 M guanidine chloride (Sigma, USA) was added to the pellet, the mixture was ultrasonicated to dissolve the
Fig. 1. (A) Schematic representation of Triticain-α. N-terminal signal peptide (amino acids 1–23) and C-terminal pro-sequence representing granulin domain (amino acids 349–461) are indicated. Predicted cysteine protease catalytic residues Cys-154, His-250 and Asn-310 are shown as C, H and N. Arrowheads point to predicted sites of the autocatalytic cleavage (Kiyosaki et al., 2009). Triticain-α-GM and its site selective mutant with Ala at position 154 [Triticain-α-GM$^{154A}$] are denoted as Ttc and Ttc', respectively. (B) SDS-PAGE of purified Triticain-α-GM (Ttc) and Triticain-α-GM$^{154A}$ (Ttc') immediately following the refolding (0 min) or after the incubation in 0.2 M sodium acetate buffer (pH 4.5) for 15 or 30 min. ‘S’ denotes the lane with molecular weight standards (the weights in kDa are indicated in the left-hand column).

protein and centrifuged for 1 h at 10,000 × g (4 °C). The supernatant was applied onto a 1.5 cm column with 5 mL nickel-nitroacetic acid (Ni-NTA) sepharose (GE Life Sciences, Sweden) equilibrated with 50 mL of 0.05 M Tris–HCl buffer (pH 8.0), 6 M guanidine chloride. The sorbent was washed with 50 mL of 0.05 M Tris–HCl buffer (pH 8.0), 8 M urea, 0.005 M imidazole and the targeted protein was eluted with 10 mL of 0.05 M Tris–HCl buffer (pH 8.0), 6 M urea, 0.25 M imidazole. After chromatography, 40 mL of 0.05 M Tris–HCl buffer (pH 8.0), 0.5 M L-arginine chloride, 2 M urea was added to the eluate and the mixture was stirred for 1 h at 4 °C. The resulting protein solution was dialyzed 3 times against 3 L of 0.01 M Tris–HCl buffer (pH 8.0) for 24 h at 4 °C. The dialyzed protein was concentrated in Amicon stirred cell supplied with a PM-10 membrane (MW cut-off 10 kDa, Millipore, USA). Protein concentration was determined using a BCA kit (Sigma, USA). The purified protein was analyzed by SDS-PAGE in 13% gel according to Laemmli (1970). Protein solution was lyophilized by freeze dryer (Jouan, France).

2.3. Enzyme activity assays

Triticain-α-GM and Triticain-α-GM$^{154A}$ autolysis/autoprocessing was monitored by incubation of 0.2 mg/mL protein in 0.2 M sodium acetate buffer (pH 4.5) at 37 °C for 15 or 30 min. The reaction was quenched by the addition of 2% SDS and boiling for 5 min and the obtained products were analyzed by SDS-PAGE.

To assess Triticain-α glutenase activity, wheat gluten (Sigma, USA) (50 mg/mL suspension in a 70% ethanol) was mixed with recombinant Triticain-α-GM (1 mg/mL in PBS) in weight ratio of 20:1 and incubated for 5 min at a variable pH (in 0.2 M glycine buffer (pH 2.6, 3.0 or 3.4) or 0.2 M sodium acetate buffer (pH 3.6, 4.0, 4.6, 5.0 or 5.6) or 0.2 M sodium phosphate buffer (pH 6.0, 6.5, 7.0 or 7.5) and temperatures (4, 16, 25, 37 or 45 °C). The reaction was quenched by the addition of 2% SDS and boiling and the obtained products were analyzed by SDS-PAGE in 14% gel. Alternatively, the reaction mixture was injected onto reverse-phase HPLC Symmetry 300 C18 column (3.9 × 150 mm, Waters, USA) and the proteolytic peptides were eluted with 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid with flow rate of 1 mL/min.

Triticain-α-GM catalyzed cleavage of collagen from bovine dermis (0.4% solution in acetic acid) was carried out exactly as gluten digestion.

To monitor Triticain-α stability in the presence of the digestive proteases pepsin and trypsin, 1 mg/mL of Triticain-α-GM was pre-incubated at 37 °C for 15 min in 0.2 M glycine buffer (pH 3.0) or 0.2 M phosphate buffer (pH 8.0) for pepsin or trypsin digestion assays, respectively. Next, 1 mg/mL pepsin from porcine stomach mucosa (Sigma, USA) or trypsin from porcine (Sigma, USA) was added and each reaction mixture was incubated at 37 °C for 15 or 30 min. The reactions were quenched by the addition of 2% SDS and boiling for 5 min and the digestion products were analyzed by SDS-PAGE in 14% gel. In control experiments bovine serum albumin (ICN, USA) was treated by pepsin or trypsin under the same conditions.

2.4. Mass-spectrometry analysis

Wheat gluten (50 mg/mL suspension in a 70% ethanol) or collagen solution (0.4% in acetic acid) was incubated with Triticain-α-GM (1 mg/mL in PBS) in weight ratio of 20:1 at 37 °C for 5 min or for more than 15 min at pH 3.4, or for more than 15 min at pH 5.6, respectively. The reaction mixture was injected onto reverse-phase HPLC Symmetry 300 C18 column and the proteolytic peptides were eluted with 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid. The total fraction of gluten/collagen proteolytic peptides was collected and its 1 mL aliquot was mixed with 0.5 µL of 2.5-dihydroxybenzoic acid saturated solution in 20% acetonitrile and 0.5% trifluoroacetic acid on the stainless steel MALDI sample target plate. Peptide masses were measured using MALDI-TOF/TOF mass-spectrometer (UltraflexXtreme Bruker Daltonics, Germany), equipped with Nd laser in positive-ion mode with a reflector. Monoisotopic masses were measured within 600–5000 m/z range with a peptide tolerance of 30 ppm. Fragment ion spectra were obtained in Lift mode. The accuracy of fragment ion mass peak measurements was within 0.5 Da. To analyze mass spectra, FlexAnalysis 3.3 software (Bruker Daltonics, Germany) was used. Gluten-derived peptides were identified based on combined MS + MS/MS data by searching for a match among plant proteins in Mascot home server NCBI protein database using Mascot search software (Matrix Science) without declaration of digest specificity. For collagen peptides the search was performed among mammalian proteins using the same conditions with additional specification for oxyprolines and oxyslines.

3. Results

3.1. Triticain-α is an autocatalytically activated cysteine protease

A central component of the complex CD treatment strategy is an enzymatic digestion of gluten-derived toxic peptides. The main goal of our study was to trial feasibility of novel proteinase from wheat Triticain-α in enzymatic therapy of the disease. To start with, it was necessary to produce recombinant enzyme possessing minimally sufficient structure and maximal activity, and to classify its catalytic type in order to subsequently
suggest the optimal conditions of its pharmaceutical application. Native Triticain-α zymogen is synthesized in wheat plantlets as 461 amino acid pro-enzyme with molecular weight of 50.4 kDa (Fig. 1A). The pro-enzyme was assumed to contain N-terminal signal peptide (amino acids 1–23) and C-terminal pro-sequence representing granulin domain (amino acids 349–461), which apparently are cleaved during the enzyme maturation (Kiyosaki et al., 2009). Therefore, to obtain functionally active recombinant enzyme, the genetic construct encoding N-terminally His6-tagged Triticain-α lacking the signal peptide and the granulin domain (hereafter Triticain-α-GM; Fig. 1A) was produced on the base of full-length Triticain-α cDNA. The obtained construct was bacterially expressed and recombinant Triticain-α-GM was purified using denaturating nickel-nitriloacetic acid (Ni-NTA) affinity chromatography. SDS-PAGE analysis of the refolded recombinant protein revealed the ~36 kDa band corresponding to Triticain-α-GM as well as three additional low molecular weight bands indicating autocatalytical processing of the enzyme. Incubation of Triticain-α-GM for 15 or 30 min in 0.2 M sodium acetate buffer solution (pH 4.5) showed further autoprocessing of the recombinant protein resulting in accumulation of the major 32 kDa product (Fig. 1B).

Based on the primary structure it was suggested that Triticain-α is a papain-type cysteine protease containing putative catalytic triad in positions 154 (Cys), 290 (His) and 310 (Asn) (Fig. 1A) (Kiyosaki et al., 2009). To test this suggestion, we obtained a mutant Triticain-α-GM with potential catalytic Cys-154 replaced with Ala (Triticain-α-GM<sup>C154A</sup>). As expected, the introduced mutation completely abrogated autoprocessing of the enzyme regardless the incubation time (Fig. 1B).

Overall, these data indicate that Triticain-α is an autocatalytically activated cysteine protease with molecular weight of the active proteolytic domain of approximately 32 kDa. Cysteine proteases are normally resistant to acid digestion and characterized by high bioavailability (Lorkowski, 2012), suggesting that Triticain-α-GM might be suitable for oral administration in enzymatic therapy. Therefore on the next stages we studied specificity of Triticain-α-GM catalyzed proteolysis and conditions favoring operation of the enzyme in terms of its potential biomedical applications.

3.2. Triticain-α exhibits glutenase activity

It was assumed that Triticain-α functions as an enzyme processing insoluble wheat grain storage proteins known as gluten to support the plantlet development (Kiyosaki et al., 2009). Considering these data, we trialed Triticain-α-GM as a potential active compound capable of performant gluten digestion which is an essential component of CD treatment. Glutenase activity

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Fig. 2. (A) SDS-PAGE of wheat gluten (Glt) incubated with Triticain-α-GM (Ttc) in weight ratio of 20:1 for 5 min at the indicated temperatures and pH values. The positions of molecular weight standards (kDa) are shown in the left-hand column. (B) Representative HPLC chromatogram confirming accumulation of multiple peptide products following incubation of wheat gluten with Triticain-α-GM in weight ratio of 20:1 at 37 °C, pH 3.4 for 5 min.
of Triticain-α was assessed under different physiologically and pharmacologically relevant conditions. Thus, wheat gluten was incubated with Triticain-α-GM in weight ratio of 20:1 for 5 min in acidic, neutral and weakly alkaline media at various temperatures. The content of resulting gluten-derived products was analyzed by SDS-PAGE or via analytical HPLC (Fig. 2). It was found that Triticain-α-GM effectively catalyzes degradation of gluten at pH 3.6, 5.6 or 6.5 and at temperatures ranging from 4 °C to 45 °C (Fig. 2A). The complete digestion was observed at acidic pH at 45 °C although at 37 °C the reaction was nearly as efficient. HPLC monitoring of Triticain-α-GM-induced gluten proteolysis under the latter conditions confirmed marked accumulation of multiple peptide products (Fig. 2B). More accurate study of Triticain-α-GM glutenase activity at 37 °C revealed its significant enzymatic potency within wide pH range between 3.0 and 6.5 (Fig. 3). It should be emphasized, that Triticain-α-GM exhibits pronounced glutenase activity at 37 °C, i.e. under human physiological conditions, and, therefore, could be used for enzymatic applications in human.

3.3. Triticain-α cleaves gluten-derived toxic peptides

The pathogenesis of CD involves inflammatory responses to digestion products of exogenous gluten. The major noxious product in this respect is a 33-mer Pro/Gln-rich peptide of α-gliadin.

![Fig. 4](image)

**Fig. 4.** (A) Triticain-α-GM cleavage sites in gluten protein components revealed by analysis of gluten-derived proteolytic peptides using MALDI TOF/TOF tandem mass-spectrometry. Asterisk indicates that more than one cleavage site of this type was identified within the substrate sequence. (B) Primary structure of α-gliadin (gi 147883566) and γ-gliadin (gi 209971907). Triticain-α-GM cleavages sites as determined by MALDI-TOF combined with tandem mass-spectrometry analysis following short-term (5 min) or long-term (>15 min) gluten proteolysis were depicted with dotted or solid arrows, respectively. X’ denotes γ-gliadin amino acids R50, T51, I52, H56 and H60 in γ-gliadin variant # gi 209971907 correspond to Q50, I51, F52, Q56 and P60 in γ-gliadin variant # gi 209971877 within short-term proteolysis peptide of the latter (46–60). The sequences of gluten-derived peptides, which are toxic for CD patients (Wieser and Koehler, 2008, 2012), are underlined. (C) Primary structure of 33-mer CD initiating α-gliadin-derived peptide (Shan et al., 2002). Triticain-α-GM cleavage sites identified following short-term (5 min) or long-term (>15 min) gluten proteolysis are indicated by dotted or solid arrows, respectively.
by MALDI TOF/TOF tandem mass-spectrometry. In order to distinguish primary cleavage sites and the sites of complete proteolysis, prior to analysis the enzymatic reaction was conducted either for 5 min or for more than 15 min. The total fractions of gluten proteolytic peptides were subjected to MS analysis whereupon the major peptide signals were selected and their fragmentation spectra were registered using MS/MS. The resulting combined spectra (MS + MS/MS) were analyzed by searching for the corresponding peptides among plant proteins and the fits exhibited highest MS score (>45) were considered as reliably identified. It was found that the short-term digestion results in formation of a number of gluten-derived fragments of which two peptides correspond to α-gliadin, five peptides derive from glutenin and low molecular weight glutenin while one peptide can be reliably referred to α-gliadin (Table S1). More prolonged gluten hydrolysis leads to accumulation of more than 30 peptides detectable by mass-spectrometry among which 13 peptides were reliably identified by MS/MS including 2 fragments of α-gliadin, 3 fragments of γ-gliadin and 8 fragments of α-gliadin (Table S1). Taken together, these data indicate that all major protein components of gluten, namely α-gliadin, γ-gliadin, α-gliadin, and glutenin, are susceptible to proteolytic digestion by Triticain-α-GM. The Triticain-α cleavage sites revealed in gluten components are summarized in Fig. 4A. Although the exact specificity of Triticain-α catalyzed cleavage is a matter of future studies, we suggest that the presence of Pro/other hydrophobic residue is being preferable in the P2 and/or P2’ positions of the substrate sequence. Remarkably, Triticain-α-GM cleavage sites were found in the majority of the previously identified gluten-derived toxic peptide sequences (Wieser and Koehler, 2008, 2012) (Fig. 4B). Furthermore, a number of cleavage sites were also observed within the sequence of 33-meric α-gliadin-derived peptide which undergoes single cleavage by Triticain-α-GM even after short-term (5 min) exposure (Fig. 4C). The latter peptide is known to accumulate as a result of peptic-catalyzed cleavage of cereal food in the stomach and initiates inflammatory response to gluten in CD patients (Shan et al., 2002). Thus our results suggest that Triticain-α is a perspective compound for medical applications aimed to perform complete gluten digestion and consequently its detoxification as a new strategy of enzymatic therapy for the CD treatment.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2015.03.001.

3.4. Triticain-α is stable under the gastric juice mimicking conditions

To date, detoxification of gluten by oral administration of glutenase(s) has been the only approved CD treatment strategy as an alternative to the gluten-free diet (Bethune and Khosla, 2012; Wieser and Koehler, 2012). Therefore, to investigate feasibility of Triticain-α-GM for oral admissions, the stability of the enzyme was analyzed under conditions simulating stomach or intestine media by focusing on pH levels and enzymatic environment. Triticain-α-GM was incubated in the presence of each of the major gastroenteric proteolytic enzymes papain and trypsin at acidic or weakly alkaline pH, respectively. Prior to experiments the activity of both enzymes was confirmed by their ability to degrade bovine serum albumin (BSA) (Fig. 5). It was found that Triticain-α-GM is relatively resistant to peptic cleavage when incubated at pH 3.0, 37°C for 30 min. Notably, under these conditions Triticain-α-GM exhibits close to maximal glutenase activity (see Fig. 2). By contrast, incubation of Triticain-α-GM in the presence of trypsin at pH 8.0, 37°C results in complete degradation of the former even within 15 min (Fig. 5). We conclude that Triticain-α-GM is stable under gastric digestion conditions wherein its capability to cleave gluten components might be substantial.

3.5. Triticain-α exhibits collagenase activity

An important current application of the enzymatic therapy is proteolysis of pathogenic collagen depositions, for instance, in wound debridement. Recently, a plant-encoded cysteine proteases GP2 from ginger was shown to possess collagenase activity (Kim et al., 2007). Therefore, to expand possible medical applications of Triticain-α-GM, its capability to hydrolyze collagen was further tested. For this purpose, Triticain-α-GM was incubated with bovine dermis collagen in weight ratio 20:1 for 5 min at various pH and temperatures. The substrate digestion and accumulation of peptide products were monitored by SDS-PAGE and HPLC (Fig. 6).
Fig. 6. (A) SDS-PAGE of bovine collagen (Col) incubated with Triticain-α-GM (Ttc) in weight ratio of 20:1 for 5 min at indicated temperatures and pH values. The molecular weights in kDa are shown in the left-hand column. (B) Representative HPLC chromatogram confirming accumulation of multiple peptide products following incubation of dermis collagen with Triticain-α-GM at weight ratio of 20:1 at 37 °C, pH 5.6 for 5 min.

Fig. 7. (A) SDS-PAGE of bovine collagen (Col) incubated with Triticain-α-GM (Ttc) in weight ratio of 20:1 for 5 min at 37 °C and indicated pH values. The molecular weights in kDa are shown in the left-hand column. (B) Triticain-α-GM cleavage sites in collagen revealed by analysis of collagen-derived proteolytic peptides using MALDI TOF/TOF tandem mass-spectrometry. Asterisk indicates that more than one cleavage site of this type was identified within the substrate sequence.
It was found that Triticain-α-GM enhances collagen cleavage at wide temperature range. The most efficient collagen digestion was observed at the highest temperature tested (45 °C) at pH 5.6 and pH 7.0 (Fig. 6A). High hydrolysis efficiency under such conditions could be associated with relaxation of collagen fibrils that might facilitate the access of the enzyme to the cleavage sites. At acidic pH values (pH 3.4) Triticain-α-GM catalyzed collagen degradation was less pronounced especially at the temperatures below 37 °C (Fig. 6A). More accurate study of Triticain-α-GM collagenase activity at variable pH at 37 °C revealed two optimal pH ranges for collagen proteolysis, namely from 3.6 to 4.6, and from 6.0 to 6.5 (Fig. 7A).

To reveal Triticain-α cleavage sites in collagen, the corresponding proteolytic peptides were studied using MALDI TOF/TOF tandem mass-spectrometry. To this end, Triticain-α-GM catalyzed collagen hydrolysis was conducted at 37 °C, pH 5.6 for more than 15 min and the resulting peptide fraction was subjected to combined MS + MS/MS analysis. The collagen fragments were identified as described above for the products of gluten digestion (see Section 3.3), except that peptide search was performed among mammalian proteins with additional specification for oxypyrolines and oxylsines which are known to be abundant in collagen substrate. More than 40 collagen-derived fragments were detected in the reaction mixture by MS among which 6 peptides were reliably identified via analysis of the corresponding fragmentation spectra (Table S2). Considering the primary structure of these peptides, 11 Triticain-α cleavage sites were found within the collagen sequence (Fig. 7B). Notably, the structure of the majority of these sites is in agreement with the rule suggested for Triticain-α-GM catalyzed proteolysis of gluten namely preferable of Pro/other hydrophobic residue in the P2 and/or P2’ positions of the substrate sequence.

Supplementary Table S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiocel.2015.03.001.

Taken together, our data indicate for the first time that Triticain-α possesses collagenase activity which is pronounced at physiologlcal pH levels at the temperature of human body. Taking into account these observations the enzyme can be considered as potential therapeutic agent in medical assays aimed to induce degradation of collagen fibrils.

4. Discussion

Insufficient and/or improper protein degradation is associated with the development of various human pathologies. For instance, pathogenesis of celiac disease (CD) involves inflammatory responses in CD patients to immunogenic peptides representing the products of digestion of insoluble grain proteins from cereal food known as gluten (Wieser and Koehler, 2008; Francavilla et al., 2014; Lundin and Solid, 2014). Being the major source of protein in human nutrition, gluten components function in plants as a seed storage proteins (Shewry and Halford, 2002; Ribeiro et al., 2013). Indeed, efficient support of seed germination and plantlet development with indispensable nutrients implicates the products of storage proteins proteolytical digestion. Recent studies report detection of mRNAs expression for a number of putative candidates for proteases functioning as glutensases in wheat germinating seeds (Kiyosaki et al., 2007, 2009; Tamura et al., 2007). Thus mRNA for a putative papain-like cystein protease Triticain-α was detected in seeds at an early stage of maturation and the stage of germination 2 days after imbibition suggesting an involvement of this protease in gluten digestion in wheat germinating seed (Kiyosaki et al., 2009). Considering these observations, in the present study we assessed glutenase and other functional activities of Triticain-α in light of its possible medical applications. To this end, recombinant enzyme lacking N-terminal signal peptide and C-terminal granulin domain (Triticain-α-GM) was obtained via bacterial expression followed by purification under denaturing conditions and refolding. Using site-directed mutageneis Triticain-α was confirmed to belong to the cysteine proteases subfamily (Fig. 1). Triticain-α-GM was shown to undergo autocatalytic activation that is in agreement with the results obtained for a number of other plant-derived papain-like cysteine proteases. Thus autocatalytic activation of proenzyme is a typical feature inherent for the members of this protein family (Bozhkov et al., 2005; Bethune et al., 2006; van der Hoorn, 2008).

As noted above, Triticain-α was predicted to function as an enzyme capable of processing storage proteins such as gluten components to support the plantlet development in wheat (Kiyosaki et al., 2009). Indeed, our in vitro studies demonstrated capability of Triticain-α-GM to efficiently hydrolyze gluten proteins (Figs. 2 and 3). Furthermore, the enzyme exhibits collagenase activity (Figs. 6 and 7). It is quite surprising since the only one plant-encoded cysteine protease, namely GP2 from ginger, was shown so far to possess this kind of activity (Kim et al., 2007). Glutenase activity of Triticain-α was detected in a broad range of temperatures at acidic or close to neutral pH levels similarly to the properties of the related glutenase EP-B2 from barley (Bethune et al., 2006). Considering the structure of Triticain-α-GM digested gluten peptides (Fig. 4), the enzyme cleavage sites were suggested to include Pro or hydrophobic residue (Leu, Phe or Ile) in P2 and/or P2’ positions of the substrate. This observation was further supported by the analysis of the products of Triticain-α-GM catalyzed collagen cleavage (Fig. 7). Similar behavior was observed for EP-B2 (Bethune et al., 2006) but otherwise the enzyme seems to differ in substrate specificity from Triticain-α. For instance, the presence of Glu at P1 or P1’ position typical for EP-B2 scarcely contributes to Triticain-α substrate specificity (Figs. 4 and 7). Although a number of sites identified within the sequences of gluten proteins contained glutamine in these positions, it can be explained by the rich content of this particular residue within gluten proteins (Fig. 4). This conclusion is supported by the observation that only 2 out of 11 identified Triticain-α cleavage sites in collagen contain Glu at P1 or P1’ positions (Fig. 7). In a similar fashion, the presence of Gly in P1 or P1’ positions of the sites of Triticain-α-GM catalyzed collagen cleavage unlikely represents a necessary criterion for the hydrolysis and could be related to the specific features of collagen primary structure consisting of GXG motif repeats. Thus broad screening experiments with a range of synthetic and/or natural substrates are needed for proper characterization of Triticain-α substrate specificity.

Based on the above encouraging results, we have explored the medicinal applications of Triticain-α. Significantly, we have demonstrated that Triticain-α-GM possesses glutenase and collagene activities at 37 °C (Figs. 2 and 6) indicating that this enzyme could be used for therapeutic assays in human. Since the enzymatic activity of Triticain-α is restricted by pH at the range from 3.4 to 6.5 (Figs. 3 and 7), it cannot be used, for instance, for protein hydrolysis in blood or intestine, whereas topical applications or oral admissions followed by the release of the enzyme into the gastric lumen are feasible. For the latter application it is important that Triticain-α was found to be relatively stable to pepsin-mediated proteolysis (Fig. 5).

Gastric, pancreatic, and brushborder proteolytic enzymes are major proteases capable of processing exogenous dietary proteins. Importantly, wheat, rye, or barley but not rice, corn, buckwheat, and potatoes are characterized by high contents of glutenine and proline, which along with other nutritionally non-essential amino acids play key roles in regulation of gene expression, cell signaling, antioxidative responses, neurotransmission, and immunity in human (Wu et al., 2013). Such amino acid composition is
typical for highly resistant to proteolysis gluten proteins, which makes the products of their partial digestion toxic to CD patients (Wieser and Koehler, 2008, 2012). Enzymatic therapy with proteolytic enzymes possessing glutenase activities was suggested as an alternative to the gluten-free diet, the only available treatment strategy approved for CD patients (Bethune and Khlosa, 2012; Wieser and Koehler, 2012; Francavilla et al., 2014). Recent report by Lähteaho et al. (2014) demonstrated successful application of a glutenase-based pharmaceutical ALV003 in phase 2 clinical trials that resulted in attenuation of gluten-induced mucosal injury in patients with CD. This represents the first experimental evidence of the efficacy of enzymatic therapy of CD in human. ALV003 is based on barley-derived glutenase EP-B2. Since glutenase activity of latter per se was insufficient for enzymatic therapy, to design ALV003 an additional prolyl endopeptidase PEP from the bacterium Sphin- gomonas capsulata was used (Siegel et al., 2006; Gass et al., 2007). Importantly, the application of relatively high doses of ALV003 was impactful: >300 mg degrade >90% of 1.5 g gluten for 30 min (Lähteaho et al., 2014). Thus new studies aimed at developing more efficient glutenases continue to be relevant. These are focused on characterization of novel bacterial enzymes such as Rothia mucilagi- nosa enzymes (Tian et al., 2014), cereal enzymes (Stenman et al., 2010), or on computational design of novel peptidases (Gordon et al., 2012). Our results obtained for novel wheat recombinant glutenase Triticain-α showed that it effectively cleaves major gluten-derived toxic peptides (Fig. 4). Thus this protease solely or in composition with other enzymes is a prospective candidate for the development of an effective pharmaceutical for the therapy of CD.

Collagenolytic enzymes were successfully applied for the treatment of a number of diseases developed as a result of pathological collagen depositions. FDA-approved class I Clostridium histolyticum collagenase-based injectable Xialflex was reported as an efficient pharmaceutical used for the treatment of Peyronie’s and Dupuytren diseases (Egui Rojo et al., 2014; Sood et al., 2014). A number of pharmaceuticals on the base of bacterial C. histolyticum, and Vibrio alginolyticus, or King Crab Paralithodes camtschatica collagenases are licensed in some countries for the use in the wound healing topically in particular for wound debridement. However there is still no conclusive opinion on preferable enzymes for this application (Glyantsev et al., 1997; Tallis et al., 2013; Onesti et al., 2014). Our data demonstrate that Triticain-α efficiently catalyzes collagen degradagtion suggesting that it can be used for instance in wound debridement. The collagenase activity of Triticain-α is limited to pH optimums (Fig. 7) enabling its topical application although implementation of the enzyme in injectable therapy is also entirely possible.

Taken together our results highlight applicability of Triticain-α in the development of novel pharmacologically active compounds of various profiles. In the following steps it is necessary to perform standardization of manufacturing processes for Triticain-α-based compounds including fermentation and purification of the active enzyme and formulation of the stable pharmaceuticals. Further, the benefit of the Triticain-α-based compounds should be verified in a set of pre-clinical studies including assessment of their ability to suppress gluten-induced inflammation in vivo (in cell cultures) and evaluation of their potency to detoxify gluten alone or in the composition with other proteases in vivo (in intact rats) (Gass et al., 2006; Stenman et al., 2010). In addition, trialing of Triticain-α-based compounds in wound healing assays using animal models is required. We believe that these studies will give rise to development of novel Triticain-α-based pharmaceuticals for CD treatment and wound debridement. Alternatively, the revealed enzymatic activities of Triticain-α can be applied in food processing industry for the production of gluten-free products or hydrolytic tenderization of meat.

5. Conclusions

In this study, we have demonstrated that the bacterially expressed recombinant wheat-derived cysteine protease Triticain-α refolds in vitro into an autocatalytically activated proteolytic enzyme, possessing glutenase and collagenase activities. Triticain-α catalyzed gluten degradation assays reveal the proteolytic digestion of the major toxic gliadin-derived peptides initiating inflammatory responses to gluten in CD patients. Analysis of the temperature and pH requirements for proteolytic activities of Triticain-α as well as its stability under the conditions simulating gastrointestinal environment show that the enzyme can be considered as a prospective medicinal candidate for gluten detoxification by oral administration in the CD treatment, whereas its collagenase activity can be employed for topical wound debridement applications.

Conflict of interest

The authors state no conflict of interest.

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