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Characterisation of gluten-degrading prolyl endoprotease from *Thermococcus kodakarensis*

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Abstract

There is increasing interest in gluten degrading enzymes for use during food and drink processing. The industrially available enzymes usually work best at low to ambient temperatures. However, food manufacturing is often conducted at higher temperatures. Therefore, thermostable gluten degrading enzymes are of great interest. We have identified a new thermostable gluten degrading proline specific prolyl endoprotease from the archaea *Thermococcus kodakarensis*. We then cloned and expressed it in *Escherichia coli*. The prolyl endoprotease was found to have a size of 70.1 kDa. The synthetic dipeptide Z-Gly-Pro-*p*-nitroanilide was used to characterise the prolyl endoprotease and it had maximum activity at pH 7 and 77°C. The V_{max} K_m and k_{cat} values of the purified prolyl endoprotease were calculated to be 3.14 mM/s, 1.10 mM and 54 s^{-1} respectively. When the immunogenic gluten peptides PQPQLPYPQPQLPY (alpha-gliadin) and SQQQFPQPQQPFPQQP (gamma-hordein) were used as substrates, the prolyl endoprotease was able to degrade these. Furthermore, gluten in wort was reduced when the prolyl endoprotease was used during mashing of barley malt. The discoveries open up for new food processing possibilities and further the understanding of proline specific protease diversity.

Introduction

Exposure to the gluten protein complex in the diet triggers celiac disease (CD) in predisposed individuals (0.5 - 1% of the population (Briani 2008; Colgrave 2011; Al-Toma 2019)), which is a chronic immune-mediated enteropathy. Gluten can be defined as a glutenin and prolamin matrix in cereals. The prolamin part in wheat is gliadin, in barley it is hordein, in rye it is secalin and in oat it is avenin (Colgrave 2011). The prolamin and glutenins are immunogenic for celiacs and non-celiac gluten sensitive people (Briani 2008; Colgrave 2011; Al-Toma 2019). Gluten activates T-cells in an immune response resulting in remodelling of tissues and malnutrition (Janssen 2015). The most effective treatment is to follow a diet based on gluten free products, which are defined as having less than 20 ppm of gluten. Gluten free foods are seen by many as beneficial and the market for them is expanding. One example is gluten free beer and an approach to lower the gluten content in conventional beers (usually made with malted barley or wheat) is prolyl endoprotease treatment. Typically it is used during the fermentation or maturation steps, which are at temperatures lower than ambient (Tanner 2013).

Prolyl endopeptidase, EC 3.4.21.26, is a proline specific endoprotease that cleaves at internal residues of proline (Walter 1980). It is closely related to other peptidases, such as oligo peptidase B (EC 3.4.21.83), dipeptidyl peptidase IV (EC 3.4.14.5) and acyl-aminoacyl peptidase (EC 3.4.19.1). These enzyme classes have therefore been studied for degrading gluten or treating celiac disease (Stepniak 2006; Mitea 2008).

The degradation of gluten peptides by microbial prolyl endopeptidase has been studied *in vitro* by several research groups (Yoshimoto 1980; Gass 2005; Lopez and Edens 2005; Riggle 2009). Janssen (2015) found that only *A. niger* prolyl endopeptidase, which is produced by DSM, could

break down immunogenic gliadin peptides from wheat. Furthermore, Walter (2014) found that only the prolyl endopeptidase from *A. niger* could result in a gluten free starch from wheat (*i.e.* which contains gliadin) and from barley (which contains hordein). Moreover, all of the enzymes discussed above are limited by the temperature and the pH range in which they are active. This makes them less suitable for food and industrial processes at elevated temperature. For example, during beer wort preparation, a sequence of stages at increasing temperature (typically *ca*. 50 °C, 64-67 °C and 72 °C) over a *ca*. 2 h period are used to produce fermentable substrates (most importantly sugars and amino acids) from the grain. This is then followed by lautering and sparging at > 70 °C for well over an hour (Bamforth 2017). Gluten degradation during the high temperature wort preparation step would be expected to result in more amino acid availability at the start of yeast growth. Recently we reported for the first time, the discovery of a prolyl endoprotease from *Sphaerobacter thermophiles* (Shetty 2017). This prolyl endoprotease had a temperature optimum of 63 °C and was capable of degrading immunogenic gluten peptides. However during beer brewing, the wort is produced over long periods above 70 °C, thus a prolyl endoprotease with even higher temperature tolerance would be beneficial.

In this study we identify and study the kinetic properties of a thermostable gluten-specific prolyl endoprotease from *Thermococcus kodakarensis*, which could be suitable for use during beer production processing steps at 70 °C or above. The gene is cloned and expressed in *Escherichia coli*. The prolyl endoprotease is then purified, characterised and the activity towards immunogenic gluten derived peptides is demonstrated.

Materials and Methods

Chemicals, substrates, strains and vectors

The prolyl endoprotease substrate Z-Gly-Pro-*p*NA was purchased from Bachem (Germany). Other

chemicals were purchased from Sigma–Aldrich (USA), except as stated in the text. *E. coli* DE3 was used as the host expression system (Invitrogen, USA). Vectors pUC57 (Initial) and pET-16b (final) were used for gene manipulations (Genescript, USA).

Recombinant enzyme cloning, culturing and expression

Possible sequences of the prolyl endoprotease family were identified in bacterial and archaeal sources using barley prolyl endopeptidase (EC 3.4.21.26) as a reference gene sequence. DNA sequence similarity searches (BLASTN) were conducted in the National Center for Biotechnology Information (NCBI) database. The prolyl endopeptidase sequence from *T. kodakarensis* was selected (UniProtKB-Q5JD37)). The nucleotide sequence was codon optimized for *E. coli* expression (see supplementary materials Figures S1 and S2) and synthesized by GeneScript (USA). The prolyl endopeptidase (PEP) gene of *T. kodakarensis* was sub-cloned into the pET-16b vector and a N-terminal His-tag and factor Xa protease cleavage site were also added. The PEP-pET-16b final vector was transformed into competent cells of *E. coli* DE3 and transformants were selected using Luria-Bertani broth (LB), containing 100 µg/mL ampicillin, at 37 °C, with shaking at 150 rpm. PEP-pET-16b transformants were subsequently cultivated under the same conditions in 40 ml of medium, in a 50 ml centrifuge tube, to an OD_{600} value of 0.6. They were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for 24 hours. The cells were harvested by centrifugation for 30 min at 10,000 g and 4 °C and the pellet was frozen at -20 °C until the prolyl endoprotease was extracted. To obtain more prolyl endoprotease, the transformants were cultivated as mentioned above, except 100 mL of medium was used in a 500 mL of Erlenmeyer flask.

Purification and characterization of the recombinant prolyl endoprotease

Stored cell pellets of the *E. coli* DE3 transformants were lysed in 10 mg/mL lysozyme (Sigma–Aldrich) in a buffer containing 10 mM NaH_2PO_4, 300 mM NaCl with pH 8.0 and they were then incubated on ice for 30 min at 4 ºC. The lysate was centrifuged at 10,000 g for 30 min at 4 ºC and then loaded onto a Ni^+–NTA spin column (Qiagen, Germany) and used according to the manufacturer's instructions. The prolyl endoprotease containing part was purified further and up-concentrated using Amicon spin columns (50 kDa cut off; Amicon, USA) as recommended by the manufacturer. Fractions were collected and examined by SDS–PAGE. The total amount of protein was determined using the Bradford method (Bradford, 1976) with reagents from Bio–Rad (USA) and bovine serum albumin (Sigma–Aldrich) as a standard. Another set of samples was prepared to further purify the prolyl endoprotease. The His-tag was cleaved off by factor Xa (Sigma –Aldrich, USA) treatment using 1 unit per mg, and then it was removed using spin columns containing Ni^+–NTA resin (Qiagen, USA) according to the manufacturer's instructions. The prolyl endoprotease portion was further purified and up-concentrated using Amicon spin columns with a cut off of 50 kDa, according to the manufacturer's protocol (Amicon, USA). The preparations were analysed for purity by SDS–PAGE.

SDS-PAGE

SDS-PAGE analysis was conducted under reducing conditions using reagents from Bio–Rad (USA). All samples were brought to the same protein concentration (1 g/L) using distilled water, then mixed with Laemmli sample buffer (4x) and reducing agent and subsequently boiled for 10 min. Samples were loaded to each SDS-PAGE gel well in volumes of 20 µL, while 10 µL of

Unstained Protein Ladder Bench Mark (Invitrogen, USA) was used as molecular marker. Bis-tris (Bio–Rad, USA) gels with 4 - 12% gradient were used and run in a tris-glycine-SDS buffer (1x) at 200 V (Thermo Scientific Pierce, USA) until the marker dye reached the end of the gel (approximately 2 h). Instant Blue (Expedeon, USA) was used for staining gels by shaking them at 50 rpm for 1 h. De-staining was carried out overnight in distilled water.

Assessment of prolyl endoprotease activity

Prolyl endoprotease activity was determined according to Edens (2005). Benzyloxycarbonyl-glycine-proline-*p*-nitroanilide (Z-Gly-Pro-*p*NA; Bachem, Germany) was used that releases nitroaniline when cleaved and which can be detected at 410 nm. A 5 mM solution of Z-Gly-Pro-*p*NA was prepared by dissolving a suitable amount in 1,4-dioxane (40% 1,4-dioxane, v/v in water) and then pre-warming to 60 °C. The standard reaction mixture contained 1000 µL of 0.1 M citrate/disodium phosphate (pH 7), with 100 µL of purified prolyl endoprotease, and 250 µL of 5 mM Z-Gly-Pro-pNA. The standard reaction conditions were to incubate the reaction mixture at 77 °C then measure in a microtiter plate reader (BioTek Synergy 2 microplate Reader (USA) using a Greiner Bio-One micro-titre plate) pre-heated to 64 °C (maximum temperature possible). For determination of V_{max} and k_m, the standard reaction mixture and standard conditions were used as defined in this section above, except that the concentration of Z-Gly-Pro-*p*NA was varied from 1 - 10 mM. One unit of prolyl endoprotease activity was defined as the release of 1 µmol of *p*-nitroaniline per minute.

Effect of pH and temperature on prolyl endoprotease activity

To assess the effect of pH on the prolyl endoprotease, it was first incubated at 77 °C for 30 min in a 0.1 M citrate-disodium phosphate buffer at pH in the range of 3 - 10. After the incubation, activity of the prolyl endoprotease was determined spectrophotometrically (410 nm) at the pH being tested, using 5 mM Z-Gly-Pro-*p*NA as a substrate and the conditions of the standard activity measurement stated above.

The effect of temperature on activity was determined by incubating the prolyl endoprotease reaction mixture at pH 7.0 for 30 min in 0.1 M citrate/disodium phosphate buffer at 20 – 100 °C. Then, the activity was measured spectrophotometrically according to the enzyme activity test conducted at optimal pH (*i.e*. 7.0) and at the temperature under test (except at 70, 80, 90 and 100 °C which were measured at 64 °C, which was the maximum temperature possible).

Thermal stability was investigated by incubating the prolyl endoprotease with 0.1 M potassium phosphate buffer for 30, 60 and 120 min at optimal pH 7.0 at various temperatures (20 30, 40, 50, 63, 70, 80, 90 and 100 °C). Then, the prolyl endoprotease activity was measured as mentioned above at pH 7.0 using the standard activity measurement procedure.

Determination of prolyl endoprotease kinetic constants

The Michaelis–Menten constants (K_m and V_{max}), and k_{cat} of the purified prolyl endoprotease were determined with Z-Gly-Pro-pNA as the substrate at concentrations of 0.2, 0.5, 1.0, 1.5, 2.0 mM under the optimum assay conditions of the standard enzyme activity method and pH 7.0. A 5.57 $mM^{-1}cm^{-1}$ extinction coefficient for nitroaniline was used at 410 nm. The reactions were started by adding 20 µL of purified prolyl endoprotease (0.398 mg/mL protein) to the buffer and substrate, with a final total volume of 250 µL in each well of a Greiner Bio-One microtiter assay plate (1 cm

light path length). Absorbance was measured at 410 nm continuously by means of a BioTek Synergy 2 microplate Reader (USA) after the addition of substrate. The kinetic data were determined using a linearised Michaelis–Menten equation (Lineweaver–Burk plot).

Effect of inhibitors and metal ions on prolyl endoprotease activity

The metal ion (at 1 mM for Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, Cu^{2+}, Mn^{2+}, Na^{+}, Co^{2+}, Fe^{2+}), inhibitor (at 1 mM for PMSF, iodoacetate) or EDTA (1, 10, or 30 mM) to be studied was added to the purified prolyl endoprotease solution and then the mixture was incubated for 30 min at 77 °C and pH 7.0. The prolyl endoprotease activity was then measured under the standard conditions and normalised with respect to a control enzyme solution without added ions, inhibitors nor EDTA.

Prolyl endoprotease activity towards immunogenic gluten peptides

The prolyl endoprotease activity towards two immunogenic peptides was assessed using: (1) PQPQLPYPQPQLPY (P1) from α-gliadin and (2) SQQQFPQPQQPFPQQP (P2) from γ-hordein as described earlier by (Geßendorfer 2011; Shetty 2017). In brief, 100 µL of peptide solution (0.2 mg/mL) and the prolyl endoprotease (50 µL) were mixed well and 10 µL of saccharin (0.4 mg/mL) was added as an internal standard. The reaction mixture was incubated at 50 °C, pH 6.5 for 90 min with continuous shaking. For inactivation, the mixture was heated to 90 °C for 10 min and then placed immediately on ice. This provided the "incubated sample". For the "inactive control", the protease was heated to 90 °C for 10 min before the substrate peptides were added. Peptide concentrations were determined by reverse phase HPLC as described by (Geßendorfer 2011). In brief, a solvent module 126 with System Gold Software (Beckman, Munich, Germany) was used with UV detector at 210 nm. A Nucleosil 100–5 C_{18}, 3 x 250 mm column was used (Macherey–Nagel, (Dueren, Germany)) at 50^{o} C. A linear gradient elution at 0.8 mL/min was used, from 0% B

to 40% B over 30 min using: (A) triethylammonium formate (TEAF) (0.01 mol/L, pH 3.0), (B) acetonitrile + TEAF (0.01 mol/L).

Sites of prolyl endoprotease cleavage of immunogenic peptides

Further analysis was carried out for cleavage of the immunogenic peptides PQPQLPYPQPQLPY and SQQQFPQPQQPFPQQP, and LGQQQPFPPQQPY, which were procured from Genescript (USA). Each peptide was dissolved in water (to give 20 mg/mL in the final reaction solution), mixed with 200 µL of prolyl endoprotease, and incubated for 3 hours at 77 °C at pH 7.0. Peptide alone served as control (*i.e.* without prolyl endoprotease) and was subjected to the same assay conditions. After incubation for 3 h, the reaction solution with prolyl endoprotease was spun through an Amicon column with 3 kDa cut off to separate the high molecular weight prolyl endoprotease from cleaved peptides. The synthetic peptide which was hydrolysed, or the control not subjected to prolyl endoprotease treatment, was brought to a concentration of 20 mg/mL before being injected onto a Superdex 200 size exclusion column (Amersham Biosciences). Water was the mobile phase at 0.5 mL/min, and an ÄKTA Purifier (PD-10, GE Healthcare) FPLC system with an automated fraction collector was used to perform the chromatography. Each individual peak was analysed by MALDI-TOF. Samples (1 µL) were loaded to an Anchor Chip target plate (Bruker Daltonics) which was followed by 1 µL of matrix solution containing 0.5 µg/µL α-cyano-4-hydroxycinnamic acid (CHCA) in 70% acetonitrile and 0.1% trifluro acetic acid (TFA). Afterwards, samples were examined by an Ultra flex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in a positive reflector mode. Flex Analysis (v3.3) from Bruker was used to analyse the spectra.

Degradation of gluten during mashing with the prolyl endoprotease

The prolyl endoprotease solution with an activity of 6.8 ± 0.14 µmol/min/mL was added to water and milled malt to give a 20 mL total volume before mashing. Two different ratios of malt to prolyl endoprotease were used. In the first, 4.5 g malt to 30.6 µmol/min prolyl endoprotease activity was used. In the second, half as much prolyl endoprotease was used, *i.e.*, 4.5 g malt and 15.3 µmol/min activity. Mashing was conducted in a Rapid Visco Analyser (RVA, Newport Scientific, Australia) using a temperature profile of 54 °C for 30 min, 64 °C for 60 min and 80 °C for 10 min. The final wort was drained through a filter paper (Whatman grade 1 qualitative), and gluten was measured using the Ridascreen R5 competitive ELISA kit (R- Biopharma, Germany). A control mashing was conducted in which no prolyl endoprotease was added. Concentrations of gliadin were calculated using the RIDA_SOFT Win software after making a standard curve with standards supplied with the ELISA kit. The results were multiplied by two, as recommended by the Codex Alimentarius Commission (2008).

Results and discussion

Prolyl endoprotease cloning and expression in E.coli

The objective of the study was to find a temperature and pH tolerant prolyl endoprotease suitable for use at temperatures above 70 °C in brewing and mashing processes. DNA sequence similarity searches (BLASTN) using barley prolyl endoprotease as a reference gene sequence were conducted in the National Center for Biotechnology Information (NCBI) database. Sequences from bacterial and archaeal sources were aligned and a phylogenetic tree was constructed focusing on thermophilic bacteria and archaea. This tree was then inspected manually and the location of *Sphaerobacter thermophilus* was determined, from which we had previously successfully cloned a

prolyl endoprotease with activity at high temperature (Shetty 2017). Microorganisms close to *S.thermophilus* were identified and the literature examined to determine if there were reports on studies with them. *Thermococcus kodakarensis* was reported as hyper theromophilic by Fukui (2005) and there were numerous other studies at high temperature with this microorganism (e.g. Atomi 2004). The 1848 bp sequence (UniProtKB - Q5JD37)) from this microorganism was therefore chosen for cloning, with the expectation that it would result in a prolyl endoprotease with very high temperature activity and stability. The gene for the putative prolyl endoprotease was cloned into *E. coli,* cultured and then the prolyl endoprotease was purified from intracellular extracts by His-tag affinity spin columns. The functional properties of the prolyl endoprotease were confirmed by the capacity to release *p*-nitro aniline from Z-Gly-Pro-*p*NA. The prolyl endoprotease had essentially the same activity before (6.8 ± 0.14 µmol/min/mL) or after (6.89 ± 0.01 µmol/min/mL) factor Xa was used to cleave off the His-tag and factor Xa digestion was not used in further work.

Over-expression of a ~70kDa protein was observed by SDS-PAGE under reducing conditions, which was purified to *ca*. 90% purity using IMAC affinity spin columns (Figure 1). Removal of the His-tag had no apparent effect on the size (Figure 1). The molecular weight of the overexpressed protein corresponds to the size expected (70.1 kDa) from the Uniprot database (UniProtKB - Q5JD37) for the *T. kodakaraensis* prolyl endoprotease (Fukui 2005). Prolyl endoproteases from other microbial hosts have been reported with a similar molecular weight (ca. 60 kDa for *Aspergillus oryzae* to 76 kDa for *Flavobacterium meningosepticum* (Kang 2014; Capiralla 2002; Yoshimoto 1980). No multimers of the overexpressed *T. kodakaraensis* prolyl endoprotease were seen and the faint band at *ca*. 14 kDa corresponds to lysozyme (theoretically 14.3 kDa) from the lysis process.

Effect of pH on prolyl endoprotease activity and stability

The prolyl endoprotease activity and optimum pH stability were determined using Z-Gly-Pro-*p*NA as substrate in a 0.1 M buffer containing citrate-disodium phosphate (pH 3 - 8) or Tris-HCl (pH 9 - 10). The data in Figure 2A show that the maximum activity at pH 7.0 was 6.9 μmol/min/mL. The pH during mashing in beer brewing is usually between 5.6 - 5.8, and here the activity of the prolyl endoprotease was reduced to *ca*. 5.8 μmol/min/mL (a 15% decrease). Previous studies of prolyl endoproteases from other microbial sources have shown many different pH optima; from 4 for *A. oryzae* (Kang 2014) to 8.7 for *Halobacterium halobium S9* (Capiralla 2002). Only that from *F. meningosepticum* showed highest activity at pH 7 (Yoshimoto 1980).

Effect of temperature on prolyl endoprotease activity

The prolyl endoprotease was incubated at various test temperatures for 30 min at pH 7.0 and then activity was measured. The activity was very stable in the range from 70 – 80 °C and had a maximum at 77 °C (Figure 2B). However, activity declined markedly as incubation temperature was raised above 80 °C and more than 50% of the activity was lost when incubated for 30 min at pH 7.0 at temperatures between 80 and 90 °C.

To test the stability of the recombinant prolyl endoprotease, it was incubated at a particular temperature for different times, before activity was measured with Z-Gly-Pro-*p*NA. The results showed that there is no enzymatic loss in activity after 2 h incubation at temperatures of up to 60 °C. Furthermore, at temperatures in the range of 70–80 °C, 90% and 85% of the recombinant prolyl endoprotease activity remained after 1 hour or 2 h incubation time, respectively (Figure 2C). For incubation under higher temperature conditions (above 80 - 90 °C), the prolyl endoprotease activity decreased by 50% and was completely lost at 100 °C, after 2 h of incubation (Figure 2C).

The prolyl endoprotease studied here has one of the highest temperature optima and stability reported so far, to the best of our knowledge. Previous studies of recombinant prolyl endoproteases have shown temperature optima which range from 30 – 45 °C. For example, the optimal temperature for prolyl endoprotease from *Aeromonas hydrophila* was 30 °C (Kanatani 1993) and for *A. niger* prolyl endoprotease it was 42 °C (Lopez and Edens 2005), whilst for *Pseudomonas* sp. KU-22 it was 45 °C (Oyama 1997). Thermal stability studies carried out on prolyl endoprotease purified from *A. oryzae* revealed that 30% activity was lost after incubation for 30 min at 50 °C and pH 4.0; however, above 55 °C prolyl endoprotease activity was lost completely (Kang 2014). In our recent work, we found a temperature optimum of 63 °C and a decline in stability when temperature exceeded 50 °C, for *S. thermophiles* prolyl endoprotease (Shetty 2017).

Effects of metal ions and selected inhibitors.

In order to gain some indications of whether metals may be important for the prolyl endoprotease activity, selected metal ions, EDTA, as well as two classic protease inhibitors were incubated with the prolyl endoprotease in 0.1 M citrate/ sodium phosphate buffer for 30 min at 77 °C and pH 7.0. There was little effect on activity when 1 mM Zn^{2+}, Co^{2+} or Fe^{2+} were tested (Supplementary results Table S1). However, activity was reduced by 10 - 15% when 1 mM of Mg^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Na^{+} or Mn^{2+} was added; 1 mM PMSF, and 1 mM EDTA led to an activity reduced by *ca*. 4 - 6%, respectively. However, 10 mM and 30 mM EDTA reduced activity by 10% (Supplementary results Table S1). Given that EDTA is renowned for its metal chelating properties, this may suggest that the prolyl endoprotease may require metal ions for its activity, but this was not investigated further. These results are partially consistent with earlier studies on prolyl endoprotease derived from *A. niger* and *A. oryzae*, which showed that Ca^{2+} ions enhanced activity significantly and, Zn^{2+} and Mg^{2+} had no effect. Inhibitory effects of Fe^{2+}, Cu^{2+}, Mn^{2} and Al^{3+} were reported by Kang

(2014) and Lopez and Edens (2005). However, the prolyl endoprotease from *A. oryzae* was inhibited by 63% by 10 mM of EDTA (Kang 2014).

Prolyl endoprotease kinetic constants

Z-Gly-Pro-*p*NA was used to determine kinetic parameters for proly endoprotease at pH 7.0. When presented in a Lineweaver−Burk plot (Figure 2D), the data gave V_{max} and K_m values of 3.14 mM/s, and 1.10 mM respectively. Given that 4.06 mg of protein was present, the specific activity was determined to be 0.773 mM/mg/s. Furthermore, k_{cat} was calculated to be 54 s^{-1} using 70.1 kDa for the molecular weight of the prolyl endoprotease and assuming that it was 100% pure.

Reduction of gluten by prolyl endoprotease during mashing of barley malt

Milled barley malt was subjected to a mashing process, with and without the prolyl endoprotease solution that has been used throughout this study. When the highest amount of prolyl endoprotease activity was used (30.6 µmol/min) it reduced the concentration of gluten by ca. 2 fold (from 590 to 290 mg/L) (Figure 3). When half as much prolyl endoprotease was used (15.4 µmol/min) the gluten content was reduced by 1.66 fold (*i.e.* to 355 mg /L). When the wort was boiled after mashing, the gluten concentrations did not change (Figure 3). A comparison can also be made with our previously discovered prolyl endoprotease from *S. thermophiles* (UniProtKB D1C7Y4), designated PEP-ST here. In that study, PEP-ST alone reduced the concentration of gluten by 3.35 fold (to 170 mg/L) when 39 µmol/min activity was tested with 4.5 g malt in 20 ml. When half as much prolyl endoprotease activity was used (19.7 µmol/min) the gluten concentration was reduced 2.24 fold to 240 mg /L (Shetty 2017). Compared to PEP-ST, the prolyl endoprotease in the current study was less efficient in degrading gluten. However, the extreme temperature stability of the prolyl endoprotease examined in the current study is advantageous compared to PEP-ST.

Prolyl endoprotease activity towards immunogenic gluten peptides

We attempted to measure the activity of the prolyl endoprotease towards two immunogenic peptides by incubating for 90 min at 50 °C at pH 6.5 and studying the fragments produced. It was found that prolyl endoprotease was highly active toward the immunogenic gluten peptides PQPQLPYPQPQLPY and SQQQFPQPQQPFPQQP. However, due to the extreme temperature stability of the prolyl endoprotease, heating to 90 °C was not sufficient to inactivate the prolyl endoprotease. Therefore, it was not possible to generate an inactive control and to calculate the specific protease activity.

Sites of prolyl endoprotease cleavage of immunogenic peptides.

For preventing celiac disease, gluten degradation should not result in immunogenic peptides. The prolyl endoprotease was therefore tested for the ability to degrade immunogenic gluten peptides. MALDI-TOF analysis was used to confirm the peptides were cleaved at the carboxyl side of the proline residue. Peptides from α-gliadin and γ-hordein that are resistant to human digestion (Shan 2002) were tested: LGQQQPFPPQQPY (α31–43), PQPQLPYPQPQLPY (α62–75) and SQQQFPQPQQPFPQQP (γ48–63). After incubation with the prolyl endoprotease, the peptide fragments were purified by FPLC (using an Äkta Purifier) and analysed by MALDI-TOF. The results (Table 1) showed that the prolyl endoprotease cleaved PQPQLPYPQPQLPY at the carboxyl side of proline residues; in particular cleaving –/P–Q and –/P–Y bonds. This gives fragments of between 1 and 13 amino acids. No fragments were found by MALDI-TOF for LGQQQPFPPQQPY or SQQQFPQPQQPFPQQP. The PEP-ST studied by us previously (Shetty 2017) showed cleavage at –/P–Q, –/P–Y and –/P–F and also generated peptide fragments of 1 to 13 amino acids.

Conclusions

An 1848 bp sequence (UniProtKB-Q5JD37) from *T. kodakarensis* is concluded to encode a proline specific prolyl endoprotease. The prolyl endoprotease has activity at very high temperatures, with a maximum activity at 77^{o}C and such high thermostability that it is difficult to completely inactivate it at 90^{o}C. This appears to be one of the most temperature tolerant prolyl endoproteases reported so far. The pH tolerance spans a broad range, which makes it suitable for food processing applications. When tested in beer wort it was capable of reducing the amount of gluten present. The prolyl endoprotease was confirmed to degrade the immunogenic peptide PQPQLPYPQPQLPY by cleaving at the internal proline residues. It is concluded that the prolyl endoprotease is a promising candidate for producing foods suitable for celiacs and non-celiac gluten sensitive people.

Conflict of interest

The authors state that they have no conflict of interest.

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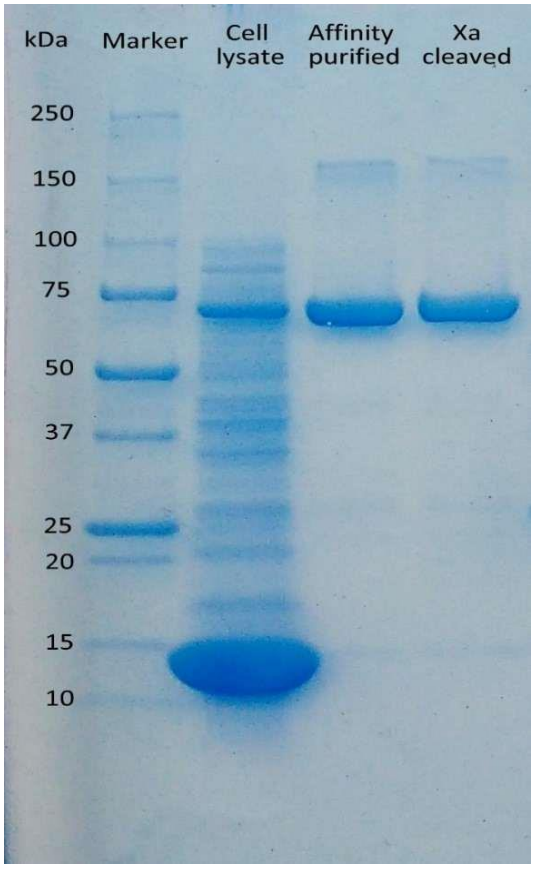

Figure 1.

SDS-PAGE (reducing) analysis of the putative prolyl endoprotease from *Thermococcus kodakarensis* expressed in *E.coli*. Lanes from left to right: Molecular weight marker; *E. coli* cell lysate; lysate following IMAC affinity purification; and after removal of the His-tag. Lysozyme is seen as a faint band at 14.3 kDa in the third and fourth lanes.

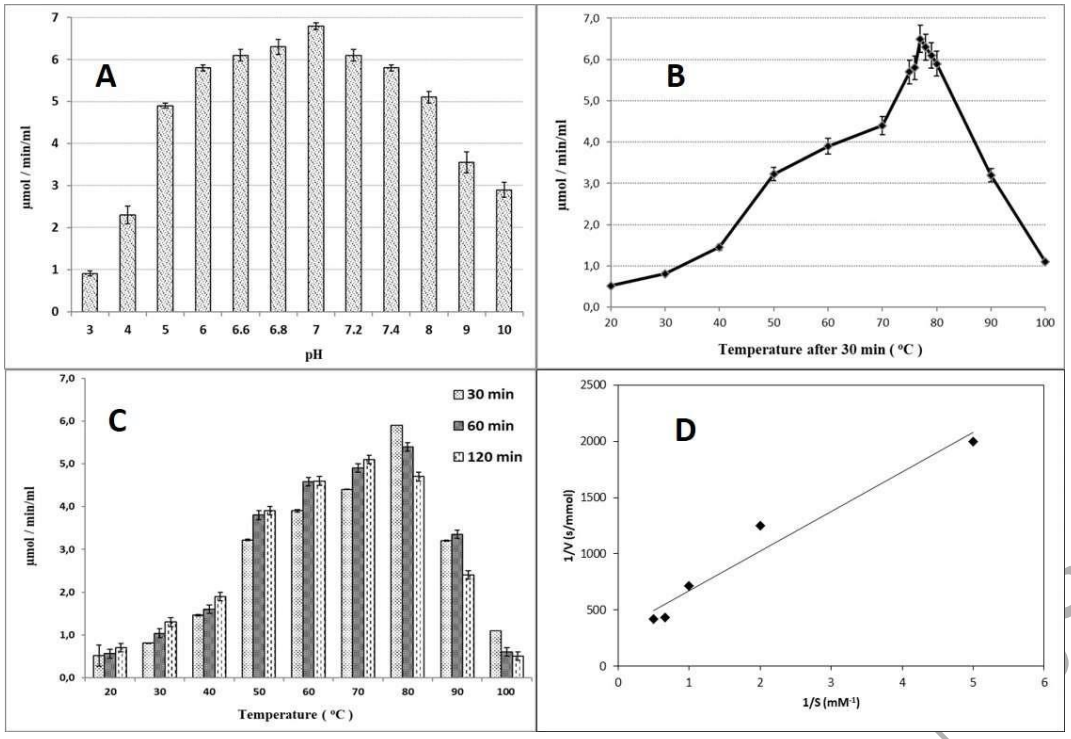

Figure 2.

A: Effect of pH on the activity of the IMAC purified prolyl endoprotease when using 1.25 mM Z-Gly-Pro-pNA substrate. The solution contained 0.398 mg/mL total protein in the reaction solution. Error bars indicate standard deviation between the duplicate experiments. B: Activity of the prolyl endoprotease after IMAC affinity purification at different temperatures. 1.25mM Z-Gly-Pro-pNA was used as substrate, and there was 0.398 mg/mL protein in the reaction solution. Following incubation at temperatures of 20-100 °C the activity was measured at 64 °C due to the maximum temperature achievable in the microtiter plate reader used. Error bars indicate standard deviation between the duplicate experiments. C: Effect of incubating at different temperatures and times on the stability of the prolyl endoprotease. 1.25mM Z-Gly-Pro-*p*NA was used as substrate and there was 0.398 mg/mL protein in the reaction solution. Following incubation at temperatures of 20 - 100 °C, the activity was measured at 64 °C which was the maximum temperature in the microtiter plate reader. Error bars indicate standard deviation between the duplicate experiments. D: Lineweaver-

Burk plot of the prolyl endoprotease with Z-Gly-Pro-pNA as a substrate ($y = 351.62x + 318.51$; $R^2 = 0.9555$)

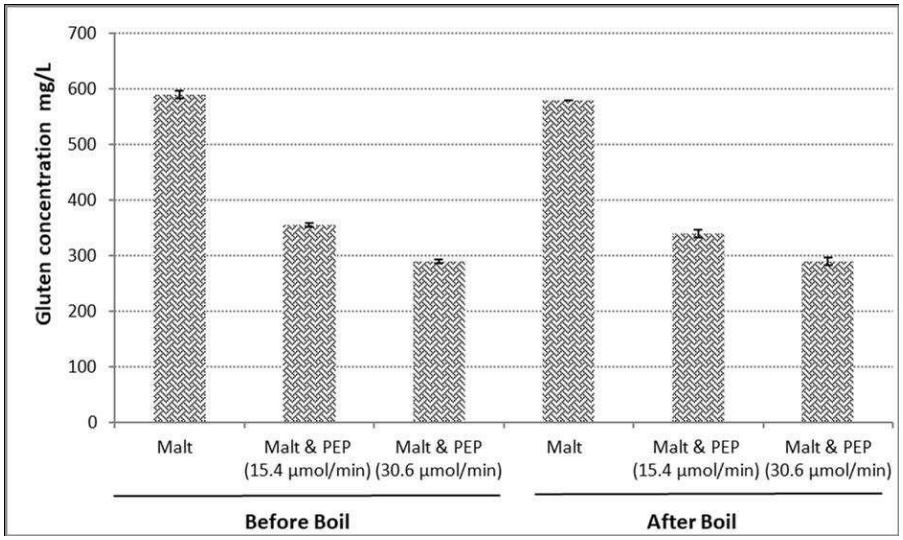

Figure 3.

Total gluten concentration in wort before boiling for 60 min at 100°C (left hand side) and after boiling (right hand side). Bars from left to right: Wort derived from malt alone, malt treated with a prolyl endoprotease activity of 30.6 µmol/min or 15.4 µmol/min.

Table 1. Peptide fragments produced by enzymatic hydrolysis of the peptide LGQQQPFPPQQPY.

Here, P: Proline; F: Phenylalanine; Q: Glutamine; Y: Tyrosine.

	Amino acid sequence	**Deduced cleavage site**
Peptide 1	L G Q QQ P F P P Q Q P Y (α31–43 gliadin)	LGQQQPFPP|QQP|Y
Identified fragments	LGQQQPFPP	–/P–Q
	LGQQQPFPPQQP	–/P–Y
	QQPY	–/P–Q
	QQP	–/P–Y
	Y	–/P–Y