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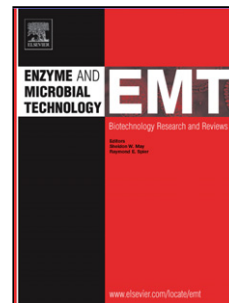
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Discovery, cloning and characterisation of proline specific prolyl endopeptidase, a gluten degrading thermo-stable enzyme from *Sphaerobacter thermophiles*

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Highlights

- High temperature stable a gluten degrading
- The ability to degrade immunogenic celiac gluten peptides
- Potential of enzyme to use during mashing to produce gluten free beer
- New insights into the effects of proline specific proteases on gluten degradation.

Abstract

Gluten free products have emerged during the last decades, as a result of a growing public concern and technological advancements allowing gluten reduction in food products. One approach is to use gluten degrading enzymes, typically at low or ambient temperatures, whereas many food production processes occur at elevated temperature. We present in this paper, the discovery, cloning and characterisation of a novel recombinant thermostable gluten degrading enzyme, a proline specific prolyl endopeptidase (PEP) from *Sphaerobacter thermophiles*. The molecular mass of the prolyl endopeptidase was estimated to be 77 kDa by using SDS-PAGE. Enzyme activity assays with a synthetic dipeptide Z-Gly-Pro-*p*-nitroanilide as the substrate revealed that the enzyme had optimal activity at pH 6.6 and was most active from pH 5.0-8.0. The optimum temperature was 63 °C and residual activity after one hour incubation at 63 °C was higher than 75 %. The enzyme was activated and stabilized by Co²⁺ and inhibited by Mg²⁺, K⁺ and Ca²⁺ followed by Zn²⁺, Na⁺, Mn²⁺, Al³⁺, and Cu²⁺. The K_m and k_{cat} values of the purified enzyme for different substrates were evaluated. The ability to degrade immunogenic gluten peptides (PQPQLPYQPQLPY (α -gliadin) and SQQQFPQPQQPFPQQP (γ -hordein)) was also confirmed by enzymatic assays and mass spectrometric analysis of cleavage fragments. Addition of the enzyme during small scale mashing of barley malt reduced the gluten content. The findings here demonstrate the potential of enzyme use during mashing to produce gluten free beer, and provide new insights into the effects of proline specific proteases on gluten degradation.

Key words: Allergy, enzyme, gluten, prolyl endopeptidase, mass spectrometry

1. Introduction

Gluten proteins have become a very important field of research within food science, due to increasing gluten intolerance including allergy issues and public concern towards gluten. Approximately 1% of the world's population suffers with celiac disease (CD) which is an autoimmune disease caused by gluten [1, 2]. Thus, there is growing focus in Denmark and worldwide on gluten free foods and beverages both for gluten intolerant and for the general public (normal people). Gluten is a common protein found in all cereals of the *triticeae* within the grass-family where barley, wheat, rye and oats are the main industrially relevant crops [3]. These cereals contain a network of large protein molecules surrounded by starch granules and other components. When the water-soluble proteins such as albumins and globulins along with starch are washed out, the remaining water insoluble protein fractions such as the prolamin and glutenin remains and is typically called gluten [3]. Gluten is thus a prolamin and glutenin matrix present in cereals and the prolamin part e.g. gliadin in wheat; hordein in barley; secalin in rye; avenin in oat [4], can cause a deadly allergic reaction in gluten intolerant people - termed celiac disease. Oat gluten is the subject of some controversy [5], but is generally considered non-allergenic.

Celiac disease occurs in the small intestine due to the ingestion of gluten and triggers an immune response by T-cells resulting in tissue remodelling and malnutrition [6]. Earlier studies by many researchers have shown that the prolamins of gluten are the toxic agent for celiac disease, since they cannot be digested appropriately in the small intestine of humans because of their specific amino acid composition. The amount of consumed gluten is therefore crucial to celiac patients [1, 2, 4]. Gluten is rich in proline which as a unique cyclic side chain structure and imparts exceptional conformational rigidity. This distinctive structural form plays an important role in the physiological condition of different diseases by protecting immunogenic peptides from proteolytic degradation [7-10]. Therefore proline is present in peptides causing various diseases such as Parkinson's disease, depression, celiac sprue, anorexia, bulimia nervosa as well as affecting blood pressure regulation. Even foods not containing whole wheat, barley, or rye often contain small amounts of prolamins from contaminations of cereals and can possibly induce allergy [11]. Gluten from these sources can be rendered safe, if it is degraded by prolyl endopeptidases into non-allergenic peptides and a number of enzyme preparations purporting to do this have come onto the market recently.

The prolyl endopeptidase (PEP), EC 3. 4. 21. 26 is also known as proline specific endoprotease and belongs to the serine protease family, has the ability to cleave peptides at internal proline residues [12]. PEP is structurally and functionally closely related to the dipeptidyl peptidase IV (EC

3.4.14.5), oligo peptidase B (EC 3.4.21.83) and acyl-aminoacyl peptidase (EC 3.4.19.1) sub-families are members of the S9 peptidase family. As such, this enzyme class has been extensively investigated for potential pharmaceutical use to degrade gluten or to treat celiacs and for other therapeutic use [13, 14].

A number of studies have examined degradation of wheat gluten and to some extent barley gluten and components by prolyl endopeptidase initially extracted from *Flavobacterium meningosepticum* [15] and other microbes, such as *Xanthomonas sp.* [16], *Aeromonas hydrophilic* [17], *Sphingomonas capsulate* [18], *Halobacterium halobium* S9 [19], *Lactobacillus helveticus* [20], *Myxococcus xanthus* [21], *Asperillus niger* [22-24] and *Aspergillus oryzae* [25]. However, these have shown ability to break down toxic gluten peptides only under *in vitro* conditions. In a very recent study Janssen et al. [6] a number of dietary supplements containing different types of gluten degrading enzymes were tested for their ability to degrade immunogenic gliadin peptides from wheat. It was found that none of these enzyme preparations could degrade the allergenic peptides, except for the *Aspergillus niger* prolyl endopeptidase (AN-PEP) produced by DSM in Holland. In another recent article by Walter et al. [26], it was reported that the PEP from *A. niger* was again found to be the only enzyme capable of delivering a gluten free starch from wheat (gliadin) and barley (hordein) and had activity 690000 times greater than from bran extracts. Stenman et al. [27] also found that enzymes from germinating barley could degrade rye secalin, eliminating toxic reactions, but few, if any studies on microbial derived enzymatic degradation of secalin, or avenin appear to have been reported. Moreover, all the enzymes discussed above are limited by the pH and temperature range in which they are active, which makes them less suitable for the majority of industrial food processes, where high temperatures are used.

The mashing process of beer brewing (e.g. 50-80 °C) is a crucial step for preparing the wort for the yeast fermentation. The mashing process takes approximately 2 hours and includes a series of rests at increasing temperature (typically ca. 54 °C, 64 °C and 78 °C) at which the various enzymes have optimal activity; which includes α -amylase, β -amylase, proteases, cellulases and β -glucanases. Subsequently there is a boiling step for 60 minutes at 100 °C before the wort is cooled and fermentation is initiated by yeast starter culture [28]. Beer brewing practices thus already involve protein degradation to produce fermentable amino acids for yeast growth, and it is not unthinkable to completely degrade gluten protein from beer during the mashing process itself. In fact, different methods of degrading and removing gluten have been developed, including the use of different proteases as well as microbial transglutaminase. However, no heat stable gluten degrading enzyme

has been reported, so current efforts focus on gluten degradation during fermentation, maturation or clarification, where temperatures are typically in the sub-zero to 20°C range. It has been reported that gluten concentration in the final beer can be reduced by use of prolyl endopeptidase during the fermentation part of the brewing process [29]. In addition, it has been demonstrated that use of acid proline-specific endoprotease from *A. niger* in low level in bottled beer prevents chill-haze formation (often caused by gluten) and had almost no effect on the beer [23]. However, gluten degradation during wort preparation would be advantageous, since it would result in more fermentable amino acids in the crucial initial stages of yeast growth.

The aim of this study was to discover and characterize a novel thermostable gluten-specific prolyl endopeptidase suitable for use during the mashing step of beer production. Subsequently to express the recombinant enzyme in *E.coli* and to demonstrate degradation of immunogenic gluten derived peptides.

2. Materials and Methods

2.1. Chemicals and reagents

Z-Gly-Pro-pNA synthetic tri-peptide substrate was used for enzyme assays (Bachem, Germany) and synthetic immunogenic celiac peptides were obtained from Genscript, USA. All other chemicals used were analytical grade which were commercially available (Sigma Aldrich, USA).

2.2. Strains and vectors

The strain *E.coli* DE3 (Invitrogen, USA) was used as host for expression. Vector pUC57 and pET-15b (Gene script, USA) were used for gene manipulations.

2.3. Cloning, culturing and expression

Enzymes likely to be proline specific prolyl endopeptidases and with potential for high temperature and suitable pH tolerance properties were obtained by screening the proteome database within Uniprot for enzymes of the class EC 3.4.21.26 and filtered by bacterial taxonomy, resulting in approximately 660 un-reviewed entries. The entries were manually investigated for heat tolerant organisms, reducing the potential entries further. From the investigated entries, the Prolyl endopeptidase from *Sphaerobacter thermophiles* was chosen (UniProtKB - D1C7Y4). The underlying nucleotide sequence was codon optimized for *E.coli* expression and synthesized (Gene Script, USA). The gene was ordered sub-cloned inside the expression vector pET-15b, where it was flanked by an in frame N-terminal His-tag, thrombin cleavage site and a start codon. The PEP-pET-15b vector was transformed into competent *E.coli* DE3 cells (Invitrogen, USA) and selected on

Lysogenic Broth or Luria-Bertani medium (LB) plates containing 100µg/mL ampicillin (Amp). PEP-pET-15b cultures were grown aerobically at 37°C at 150 rpm in LB 100µg/mL Amp. Cultures were grown to an OD₆₀₀ of 0.6 and induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 24 hours the cultures were centrifuged at 10,000 g for 30 min at 4 °C and stored at -20 °C as cell pellets until later use. For production of larger amounts of enzyme, cultivations were as described above, except using 100 ml of medium in a 500 ml Erlenmeyer flask.

2.4. Purification and characterization PEP

Cell pellets were dissolved in 10mg/ml lysozyme (Sigma–Aldrich, USA) in a 10 mM NaH₂PO₄, 300 mM NaCl, pH 8.0 buffer and incubated at 4 °C for 30 min on ice. The resulting lysate was centrifuged at 10,000 g for 30 min at 4 °C and applied to a Ni⁺–NTA resin spin column (Qiagen, USA) under non-denaturing conditions according to the manufacturer’s recommendations (Qiagen, USA). The enzyme fraction was further purified and concentrated using Amicon spin columns with 50 kDa cut off according to manufacturer’s recommendations (Amicon, USA). The different fractions during the process were retained and analysed by SDS–PAGE. The total protein concentration was determined by the Bradford method [30] using Bradford reagent (Bio-Rad, USA) and by using bovine serum albumin (Sigma–Aldrich, USA) as standard. The his-tag was also removed by treatment with thrombin, 1 units per mg (Sigma Aldrich, USA), and applied to Ni⁺–NTA resin spin columns (Qiagen , USA). The enzyme fraction was further purified and concentrated using Amicon spin columns with 50 KD cut off according to the manufacturer (Amicon, USA) as mentioned above. Both final fractions containing the desired enzyme with his-tag and thrombin cleaved enzyme were checked for purity by SDS–PAGE.

2.5. SDS-PAGE analysis

Reducing SDS-PAGE was carried out according to Laemmli et al. [30] as described previously by Studier [31]. All samples used had the same protein concentration (1g/L) and were mixed with Laemmli sample buffer (4x) with reducing agent (Bio-Rad, USA) and boiled for 10 min. 20µL were loaded per samples, while 10µL of Bench Mark™ Unstained Protein Ladder (Invitrogen, USA) were loaded for each ladder. 4-12 % Bis-Tris (Bio-Rad, USA) gels were run at a 200V in a Tris-glycine-SDS buffer (1x) (Thermo Scientific Pierce, USA). Staining was done with Instant Blue (Expedeon, USA) with slow shaking (ca. 50 rpm) for 1 hour, de-staining was done overnight with water.

2.6. Determination of prolyl endopeptidase activity

Prolyl endopeptidase enzyme activities were determined according Eden et al. [22] using the synthetic chromogenic peptide substrate benzyloxycarbonyl-glycine-proline-*p*-nitroanilide (Z-Gly-Pro-*p*NA; Bachem, Germany), which releases nitroaniline that can be detected at 410 nm. Z-Gly-Pro-*p*NA substrate was dissolved in 1,4-dioxane (40 %, v/v in water) at 60 °C to prepare a 5 mM solution. For the standard reaction mixture, there was 1000 µl of 0.1 M citrate/disodium phosphate buffer (pH 7), 100 µl of purified enzyme, and 250 µl of 5 mM Z-Gly-Pro-*p*NA. This was used at 60 °C unless mentioned otherwise. For determination of V_{\max} and k_m , the standard reaction mixture and conditions were used, except the concentration of Z-Gly-Pro-*p*NA was varied from 1 – 10 mM. One unit of prolyl endopeptidase activity was defined as the release of 1 µmol of *p*-nitroanilide per minute under assay conditions. Controls were made at the temperature and conditions under test to ensure there was no spontaneous substrate hydrolysis, by replacing the enzyme sample with distilled water.

2.7. Characterization of pH and temperature dependency of the recombinant PEP

2.7.1. pH optima

To estimate the optimum pH, purified enzyme was first incubated in 0.1 M citrate/disodium phosphate buffer at pH ranging from pH 3 to 10 at 60 °C for 30 min. After incubation, the prolyl endopeptidase activity was measured (at 60 °C and at the pH being tested) at 410 nm spectrophotometrically using 5 mM Z-Gly-Pro-*p*NA as substrate as mentioned earlier.

2.7.2. Temperature optima

Thermal dependence of PEP activity was determined by incubating the reaction mixture of enzyme in 0.1 M citrate/disodium phosphate buffer at different temperatures from 20 to 80 °C for 30 min at pH 6.6. Then, the activity was determined according to the enzyme activity test at optimal pH 6.6 and at the temperature under test (except for test temperatures of 70 and 80 °C which were measured at 64 °C due to the equipment available).

2.7.3. Thermal stability studies

Thermal stability assay was conducted by incubating PEP enzyme solution in 0.1 M potassium phosphate buffer (optimal pH 6.6) for 5, 30, 60 and 120 min at different temperatures (10, 20 30, 40, 50, 63, 70 and 80 °C). Then, the activity was determined as mentioned earlier at pH 6.6 and 64 °C.

2.8. Enzyme kinetics PEP

Enzyme kinetics Michaelis–Menten constant (K_m), maximal velocity (V_{max}), and k_{cat} of the purified enzyme were determined using Z-Gly-Pro-pNA with substrate concentrations in the reaction mixture of 0.2, 0.5, 1, 1.5, 2 mM under the optimal assay conditions in the standard method, *i.e.* 60 °C and pH 6.6. An extinction coefficient for nitroaniline of $5.57 \text{ mM}^{-1}\text{cm}^{-1}$ at 410 nm was used. The reactions were conducted by adding 20 μL of enzyme (containing 0.208 mg/ml protein) to buffer and substrate and the final volume was 250 μL in a microtitre plate well, which equated to a 1 cm path length. Absorbance was measured constantly by a BioTek Synergy 2 microplate Reader (USA) after substrate addition using a Greiner Bio-One micro-titre plate. The kinetic data were calculated from Lineweaver–Burk plots using the Michaelis – Menten equation.

2.9. Effect of metal ions on enzyme activity

1 mM of each metal ion (Mg^{2+} , Ca_2^+ , K^+ , Cu^{2+} , Zn^{2+} , Na^+ , Mn^{2+} , Co^{2+} , Fe^{2+}) or the inhibitors Iodoacetate (1 mM), PMSF (1 mM) and EDTA (1, 10, 30 mM) was added to the purified enzyme solution and incubated for 30 min at 63 °C and pH 6.6. Subsequently enzyme activity was determined under the standard condition (60 °C, pH 6.6). Enzymatic activities were expressed as relative values (%), with enzyme not containing added ions or inhibitors serving as control (*i.e.* 100 % activity).

2.10. Determination of Prolyl endopeptidase activity on gluten-specific synthetic immunogenic peptides

The activity towards immunogenic gluten epitopes was determined as described by Gessendorfer et al. [32] using two celiac disease-active peptides PQQQLPYPQQQLPY (P1) from α -gliadins and SQQQFPQQPFPQQP (P2) from γ -hordeins. Peptide solution (100 μL , 0.2 mg/mL) and peptidases PEP (50 μL) were mixed and saccharin (10 μL ; 0.4 mg/mL) was added as an internal standard. The mixture was incubated at 50 °C and pH 6.5 under continuous shaking in a heated thermomixer (HTML-133, HLC, Bovenden, Germany) for 90 min. The reaction was stopped by increasing the temperature to 90 °C for 10 min (this is donated as the incubated sample). In the non-incubated sample, peptidases were inactivated by heating prior to incubation. Samples were centrifuged (20 min at $12,846 \times g$; Biofugepico, Heraeus, Hanau, Germany) and analyzed by RP-HPLC. Samples (40 μL) were separated on a Thermo Finnigan Spectra System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with *Chrom Quest* software using a Nucleosil C18 column (3 x 250 mm, 5 μm , 10 nm; Macherey-Nagel, Dueren, Germany) at room temperature. Conditions: Solvent A, 0.1 % (v/v) trifluoroacetic acid (TFA); solvent B, 0.1 % (v/v) TFA in acetonitrile; gradient, 0 - 10 min linear 0 - 37.5 % B, 10 - 14 min isocratic 37.5 % B, 14 - 15 min

linear 37.5 - 90 % B, 15 - 20 min isocratic 90 % B. The flow rate was 0.7 mL/min and the effluent was monitored at 210 nm. The HPLC peak areas of P1 and P2 were determined for the non-incubated and the incubated samples. The time-dependent decrease of the absorbance areas was used to calculate the peptidase activity in U/ μ L.

2.11. Examination of cleavage pattern on synthetic immunogenic peptides

In a second analysis for cleavage of immunogenic peptides, two of the synthetic peptides used above (i.e. PQPQLPYPQPQLPY and SQQQFPQPQQPFPQQP), as well as LGQQQPFPPQQPY were employed and were obtained from Gene script, USA. Here 20 mg/ml of the peptide under study was dissolved in water and incubated with 200 μ L of PEP enzyme at a temperature of 63 °C and pH 6.6 for 3 hours. A control with peptide alone (i.e. without PEP) was also made and subject to the same assay conditions. After incubation, the reaction solution was spun through an Amicon 3 kDa cut off columns to remove the high molecular weight enzyme. The hydrolysed synthetic peptide or the control without enzyme treatment were adjusted to a concentration of 20 mg/ml and injected into an ÄKTA purifier (PD-10, GE Healthcare) with 10 kDa cut off size exclusion column (Superdex 200, Amersham Biosciences). Water was used as equilibrium and elution buffer with a flow rate of 0.5 ml/min and eluents were collected in an automated fraction collector. Fractions from individual peaks were pooled and analysed by MALDI-TOF. Briefly, 1 μ L samples were loaded on an Anchor Chip target plate (Bruker Daltonics) followed by 1 μ L of 0.5 μ g/ μ L matrix solution (α -cyano-4-hydroxycinnamic acid (CHCA) in 70 % ACN, 0.1 % trifluoro acetic acid (TFA)). Samples were analysed using an Ultra flex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in positive reflector mode and spectra were processed by Flex Analysis (v3.3) software provided by the instrument manufacturer.

2.12. Effect of the PEP during mashing on gluten from malt

To check the ability of the PEP enzyme to degrade gluten under mashing conditions, the enzyme solution (activity ca. 8.77 ± 0.08 μ mol/min/ml) was mixed with milled malt at a ratio of 1:1 (i.e. 4.5 g malt and 4.5 ml of enzyme solution, plus water to give a total volume of 20 ml), or 1:0.5 (i.e. 4.5 g malt : 2.25 ml enzyme plus water to give 20 ml) in a rapid visco analyser (RVA, Newport Scientific, Australia). Afterwards a pre-established mashing temperature profile [39], 2010) was used as follows: 54 °C and rest for 30 min; heating up to 64 °C and rest for 60 min; a further heating up to 80 °C and rest for 10 min. The wort was drained through WhatmanTM grade 1 qualitative filter paper, samples were collected and total gluten content was measured using a Ridascreen R5 competitive ELISA kit (R- Biopharma, Germany) according to the manufacturer's

instructions. Mashing with the enzyme solution replaced with an equivalent volume of water served as control. Gliadin concentrations were calculated based on a cubic spline calibration function with standards supplied with the ELISA kit and by using the RIDA_SOFT Win software (R-Biopharm, Germany). The resulting values were converted into gluten concentrations by multiplying by a factor of two (Codex Alimentarius Commission, 2008).

3. Results and discussion

3.1. Cloning and expression of prolyl endopeptidase

Given that the aim was to discover a new high temperature and pH tolerant prolyl endoprotease, the proteome database within Uniprot was explored for enzymes from heat tolerant bacteria. An open reading frame composed of 2064 bp, (UniProtKB - D1C7Y4) was found in *Sphaerobacter thermophiles*, which is a bacterium that has been reported to grow in the temperature range from 40-65 °C [33]. The DNA encoding this putative prolyl endopeptidase gene was then inserted into a vector behind the sequence for a poly histidine tag and thrombin cleavage site in order to facilitate subsequent isolation of the enzyme. Cells from transformed *E.coli* strains expressing the recombinant enzyme were harvested and the enzyme was purified from intracellular extracts by IMAC affinity spin columns. The functionality of the PEP was confirmed by its ability to hydrolyze Z-Gly-Pro-pNA to release *p*-nitro aniline which was measured at 410 nm spectrophotometrically. The purified PEP enzyme was active both before (8.77 ± 0.08 $\mu\text{mol}/\text{min}/\text{ml}$) and after (8.85 ± 0.04 $\mu\text{mol}/\text{min}/\text{ml}$) cleaving with thrombin. Given there was no significant difference in activity, we omitted thrombin digestion in further work.

A reducing SDS-PAGE analysis showed over-expression of a protein of ~78 kDa, which was purified to ca. 90 % purity by the IMAC affinity step (Fig. 1). The histidine tag was subsequently cleaved off the PEP enzyme using thrombin which delivered a protein with a molecular weight of approximately 77 kDa (Fig. 1). No multimers were seen. The contaminating band at ca. 14 kDa (Fig 1) was not isolated or characterised, but probably corresponds to lysozyme (theoretically 14,3 kDa) added in order to disrupt the cells. The size of 77 kDa is similar to the molecular weight of prolyl endopeptidase predicted (76.6 kDa) (UniProtKB - D1C7Y4) from the complete genome sequence of *Sphaerobacter thermophiles* [34]. Previously reported prolyl endopeptidases have similar molecular weights, e.g. from *Aspergillus oryzae* (60 kDa), *Aspergillus niger* (66 kDa), *Halobacterium halobium* S9 (71 kDa), *Sphingomonas capsulata* (75 kDa), *Pseudomonas* sp. KU-22 (76 kDa), *Aeromonas hydrophila* (76.4 kDa), *Xanthomonas* sp. (75 kDa), *Flavobacterium meningosepticum* (76 kDa) and Human brain (79 kDa) [15-19, 23, 35-37].

pH optima and stability

The optimal pH of the PEP enzyme in terms of activity and stability was examined using Z-Gly-Pro-pNA as substrate in 0.1 M citrate/disodium phosphate buffer (pH 3-8) or Tris-HCl (9-10). The results in figure 2 show the highest activity at pH 6.6. At the typical mashing pH of 5.6-5.8, the activity was still very high and had only declined to ca. 7.5 U from 9.4 U. Previous studies of prolyl endopeptidases showed various pH optima and pH stability. PEP extracted from *Aspergillus oryzae* [35] and *Aspergillus niger* [23] showed maximum activity at pH 4 and 4.2 and was stable at pH 3-5. On the other hand, other prolyl endopeptidases had maximum activity at alkaline pH, for example *Halobacterium halobium* S9 (pH 8.7) [19], *Sphingomonas capsulata* (pH 8.5) [18], *Pseudomonas* sp. KU-22 (pH 8) [36], *Xanthomonas* sp. (pH 7.7) [16] and *Aeromonas hydrophila* (pH 7.7) [17]. *Flavobacterium meningosepticum* had highest activity at pH 7 [15] and Human brain at 6.8 [37].

3.2. Temperature optima and thermal stability of PEP

The effect of temperature on activity was examined by incubating the enzyme at the test temperature for 30 min at pH 6.6, then adding substrate and measuring activity. The PEP activity was strongly stimulated upon temperature increase and the highest activity was observed at 63 °C (Fig. 3). Importantly, high activity was seen in the temperature range 50-75°C (Fig. 3) where the longest time is spent during beer mashing. The PEP enzyme activity was very stable between the temperatures ranging from 50-63 °C after incubation for 30 min. The activity decreased rapidly at temperature above 63 °C. About 65 % activity of the enzyme was lost when enzyme is exposed to 70 to 80 °C for 30 min at pH 6.6.

To test the effect of temperature on stability of the enzyme, it was incubated at the test temperature for different times, then Z-Gly-Pro-pNA was added and activity measured. The results showed that no activity was lost after 2 h incubation up to 30°C. However at temperature between 40-63 °C, 30 % of the PEP enzyme activity was lost upon incubation for an hour and a further 50 % lost after 2 hours of incubation (Fig. 4). Whereas, at higher temperature (70 and 80 °C), the enzyme activity was completely lost, after 2 hours of incubation (Fig. 4). Comparison with the literature suggests the PEP characterised in the current work has the highest temperature optimum and stability reported so far. Previous studies of prolyl endopeptidases showed temperature optima ranging from 30-50 °C. For example, prolyl endopeptidase from *Aspergillus niger* had optimal temperature of 42 °C [23], *Pseudomonas* sp. KU-22 was 45 °C [36], *Halobacterium halobium* S9 was 40 °C [19], *Sphingomonas capsulata* was 43 °C [18], *Flavobacterium meningosepticum* was 40 °C [15], A.

hydrophila was 30 °C [17] and human brain PEP was 37 °C [37]. Thermal stability studies of purified prolyl endopeptidase from *Aspergillus oryzae* showed it was stable up to 50 °C with a residual activity of above 70 % after incubating at pH 4.0 for 30 min but enzyme activity was lost above 55 °C [35].

3.3. Effects of metal ions and inhibitors

It is known that some enzymes contain metal binding sites and it is possible that the PEP enzyme activity may be influenced positively or negatively by addition of metal ions and inhibitors. To test this, the enzyme along with metal ions, inhibitors or EDTA were incubated with 0.1M citrate/sodium phosphate buffer for 30 min at 60 °C and pH 6.6 and activities were measured using Z-Gly-Pro-pNA. The metal ions Co^{2+} (1 mM) and Fe^{2+} (1 mM) had almost no effect on enzyme activity (2 % or less increase in relative enzyme activity). In contrast, 1 mM of Mg^{2+} , Ca^{2+} , K^+ , Cu^{2+} , Zn^{2+} , Na^+ and Mn^{2+} reduced enzyme activity approximately 10 -15 %. The inhibitors iodoacetate (1 mM), PMSF (1 mM), and 1 or 10 mM EDTA had little effect on activity (i.e. ca. 4 % lower), whereas 30 mM EDTA reduced activity by 10 % suggesting that the PEP may require metal ions for activity, but this was not investigated further (Table. 1). Previous studies on PEP from *Aspergillus oryzae* and *A. niger* showed that Ca^{2+} ions significantly enhanced prolyl endopeptidase activity whereas, Zn^{2+} and Mg^{2+} did not have any effect; and Fe^{2+} , Cu^{2+} , Mn^{2+} and Al^{3+} showed inhibitory effects [23, 35]. *Aspergillus oryzae* prolyl endopeptidase was slightly affected (within 5 %) by 1 mM PMSF (98 ± 2.5 relative enzyme activity (%)) or 1 mM EDTA (95.2 ± 0.5 relative enzyme activity (%)) and was inhibited (63 %) by 10 mM of EDTA (37.3 ± 0.5 relative enzyme activity (%)) [35]. Diisopropylfluorophosphate (DPF) has also been shown to inhibit a range of other PEPs [15-19, 23, 35-37].

3.4. Determination of V_{\max} , K_m and K_{cat}

Kinetic parameters for the *S. thermophiles* PEP were determined at pH 6.6 and 63 °C with the Z-Gly-Pro-pNA substrate. The results gave a good fit to the Lineweaver Burk plot (Fig. 5) allowing determination of V_{\max} and K_m to be 3.47 mM/s, and 1.5 mM respectively, when 4.16 mg of protein was present in the reaction solution, i.e. the specific activity is 0.834 mM/mg/s. Given that the molecular weight of the enzyme was found above to be 77 kDa and assuming that it was 100 % pure, then k_{cat} can be estimated to be 60 s^{-1} .

3.5. Effect of PEP on gluten content during mashing

Total gluten content was determined following mashing with milled barley malt and the PEP

enzyme solution (i.e. with the same activity which has been used in all the studies in the current work) added in the same ratio as the malt (1:1 ratio) or at half the volume of the malt (1:0.5). Malt without enzyme served as control. The PEP enzyme significantly reduced the gluten content by 3.35 fold (to 170 mg/L) when used at the 1:1 ratio with malt, whereas using half as much enzyme (1:0.5 ratio) reduced gluten only by 2.24 fold (i.e. to 240 mg /L), compare to control malt alone that had 570 mg/L gluten (Fig. 6). Wort boiled for one hour after mashing showed no further significant reduction in gluten (Fig. 6). Although the gluten was not reduced to 20 mg gluten/kg (i.e. “gluten free”) or to 100 mg gluten/kg (i.e. “low gluten level”) the results do confirm gluten degrading ability of the enzyme under the high temperature and low pH condition during mashing. These results are therefore consistent with earlier work by Weisner and Koehler [41] which we reviewed showing that PEP enzymes can degrade proteins as well as peptides. Therefore a large scale production of the enzyme discovered here to allow higher enzyme loadings, possibly in combination with an exopeptidase or transglutaminase should be examined in the future.

3.6. Confirmation that the PEP degrades antigenic gluten peptides.

We have demonstrated that the PEP discovered here has activity towards simple substrates and can degrade gluten in beer wort as demonstrated by ELISA. However, there has been discussion in the literature that even though gluten may be degraded, immunogenic peptides may remain. We therefore tested the ability of the PEP to degrade known antigenic peptides. This was first done by incubating the antigenic peptide under study for 90 min at 50 °C at pH 6.5 and measuring the fragments produced. Endopeptidase was highly active toward both immunogenic gluten peptides PQPQLPYPQPQLPY and SQQQFPQPQQPFPQQP. The enzyme showed gluten-specific peptidase activities of 4.7 ± 0.6 Units/ μ L against α -Gliadin (p62–p75; PQPQLPYPQPQLPY) and 1.6 ± 0.2 Units/ μ L against γ -Hordein (p48–p63; SQQQFPQPQQPFPQQP).

A peptide cleavage assay study with analysis by MALDI-TOF was also conducted to demonstrate that the enzyme cleaved at the carboxyl side of proline residues in celiac disease active peptides. The peptides from α -Gliadin (α 31–43) LGQQQPFPPQQPY, α -Gliadin (p62–p75) PQPQLPYPQPQLPY and γ -Hordein (p48–p63) SQQQFPQPQQPFPQQP which is resistant to human digestive enzymes [38] were incubated along with PEP. Purification by FPLC and identification of these resulting fragments by MALDI-TOF (Table. 2) showed that PEP successfully cleaved these gluten peptides at the carboxyl side of proline residues. In particular at -/P-F, -/P-Q and -/P-Y peptide bonds were cleaved which resulted in peptide fragments of 1 - 13 amino acids. We are able to see fragments of 7 or above 7 amino acids with MALDI-TOF.

Previous reports have suggested that peptides larger than 8 amino acids may still cause celiac disease, therefore further work should be done to examine the allergenic effects of different small gluten peptides, but this is outside the scope of the current work. Nevertheless, the results found here from the study on gluten degradation show that the enzyme degrades

4. Conclusions

The present study reveals an open reading frame composed of 2064 bp, (UniProtKB - D1C7Y4) from *Sphaerobacter thermophiles* encoding a gene with proline specific prolyl endopeptidase activity. When expressed in *Escherichia coli* and isolated, it was found to have a broad range of pH stability and high temperature activity and stability. The enzyme degrades the celiac-active peptides we tested, i.e. α -gliadin LGQQQPFPPQQPY, PQQQLPYPQQQLPY and γ -hordein SQQQFPQQPFPPQQP by cleaving at internal proline residues. It is one of the few PEP enzymes which have been confirmed to have such activity. It is thus a promising enzyme for applications where gluten degradation at high temperatures offers processing advantages, for example to degrade and reduce gluten during the high temperature mashing process of beer brewing.

Conflict of interest

The authors all declare that they have no conflict of interest.

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Figures captions

Fig. 1: Reducing SDS-PAGE of expression and purification of recombinant putative PEP from *S.thermophiles*. Lanes from left to right show; molecular weight marker; *E.coli* cell lysate before treatment; after IMAC purification; and following treatment with thrombin and subsequent removal of released tags by size exclusion. Thrombin if present would be composed of two chains at 31 kDa and 6 kDa; lysozyme used in the lysis mixture is seen at 14.3 kDa.

Fig. 2: Activity of the putative PEP as a function of pH with 1.25 mM Z-Gly-Pro-pNA as a substrate. There was 0.421 mg/ml protein in the reaction solution and it had been affinity purified by his-tagged columns.

Fig. 3: Effect of temperature on the activity of the putative PEP with 1.25 mM Z-Gly-Pro-pNA as a substrate. There was 0.421 mg/ml protein in the reaction solution. Following incubation at temperatures of 65 °C and above the activity was measured at 64 °C due to limitations in the microtitre plate system available.

Fig. 4: Effect of temperature on the stability of the PEP examined with 1.25 mM Z-Gly-Pro-pNA as a substrate. There was 0.421 mg/ml protein in the reaction solution. Following incubation at temperatures of 65 °C and above the activity was measured at 64 °C due to limitations in the micro titre plate reader system available

Fig. 5: LineweaverBirk plot of the PEP enzyme with Z-Gly-Pro-pNA as a substrate.

Fig. 6: Total gluten content estimation by Ridascreen R5 competitive ELISA in (from left to right) wort derived from malt alone, malt treated with PEP enzyme in volume ratio of malt: enzyme of 1:0.5 and 1:1 ratio. Also another set of samples are collected after 1 hour of boiling at 100 °C mimic brewing condition.

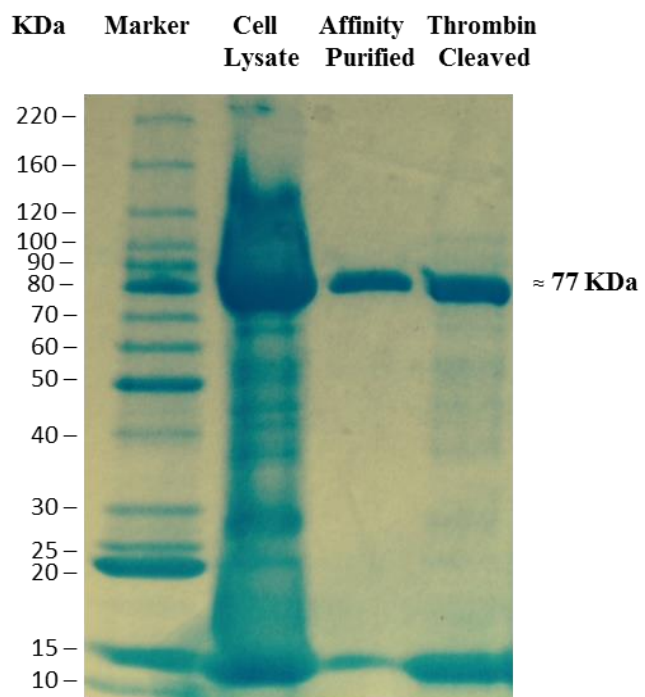


Fig. 1.

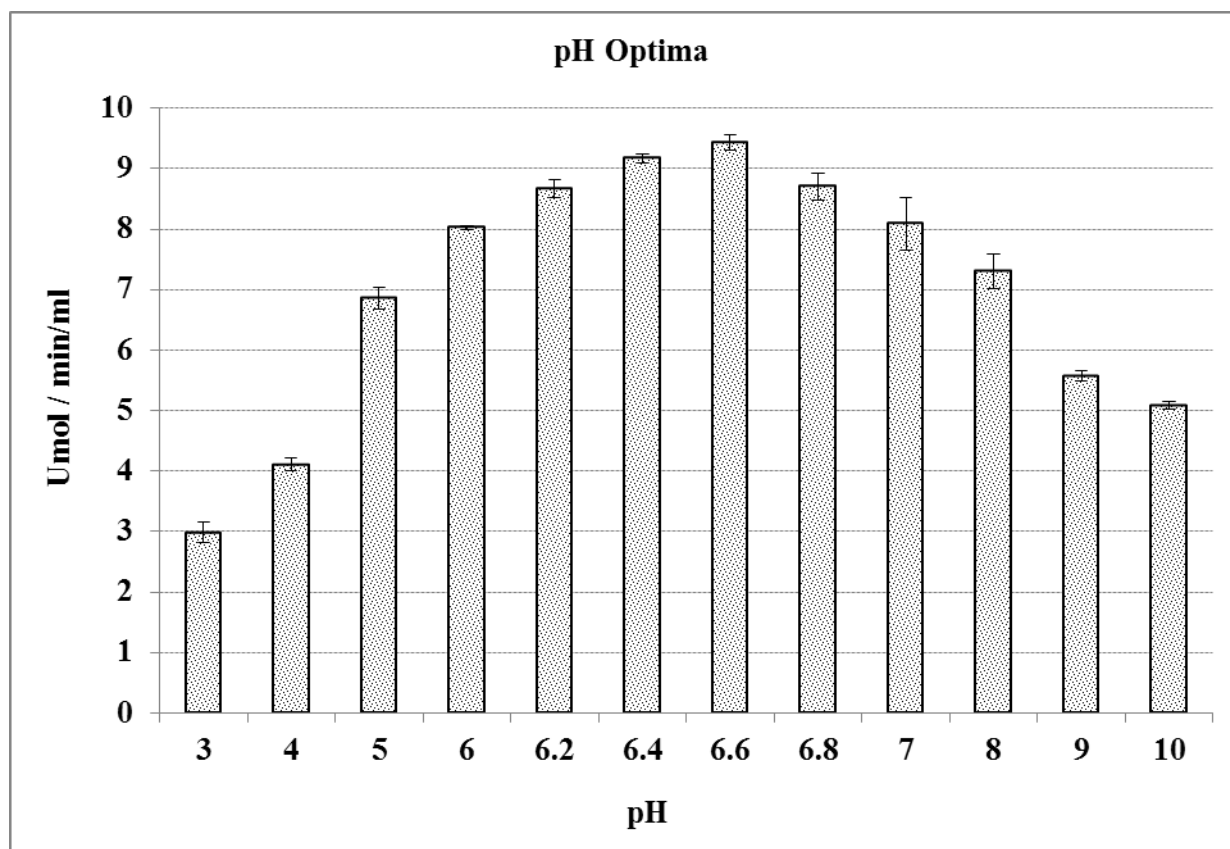


Fig. 2.

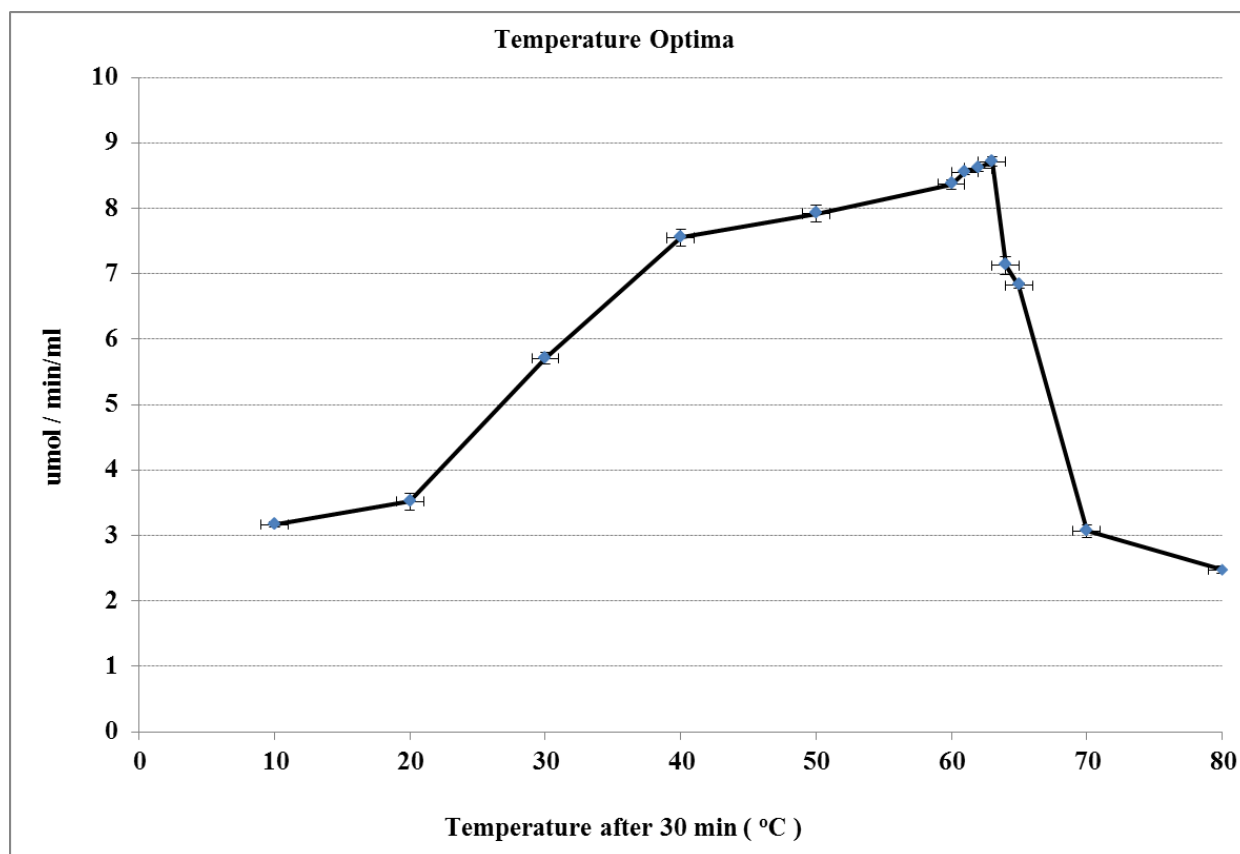


Fig. 3.

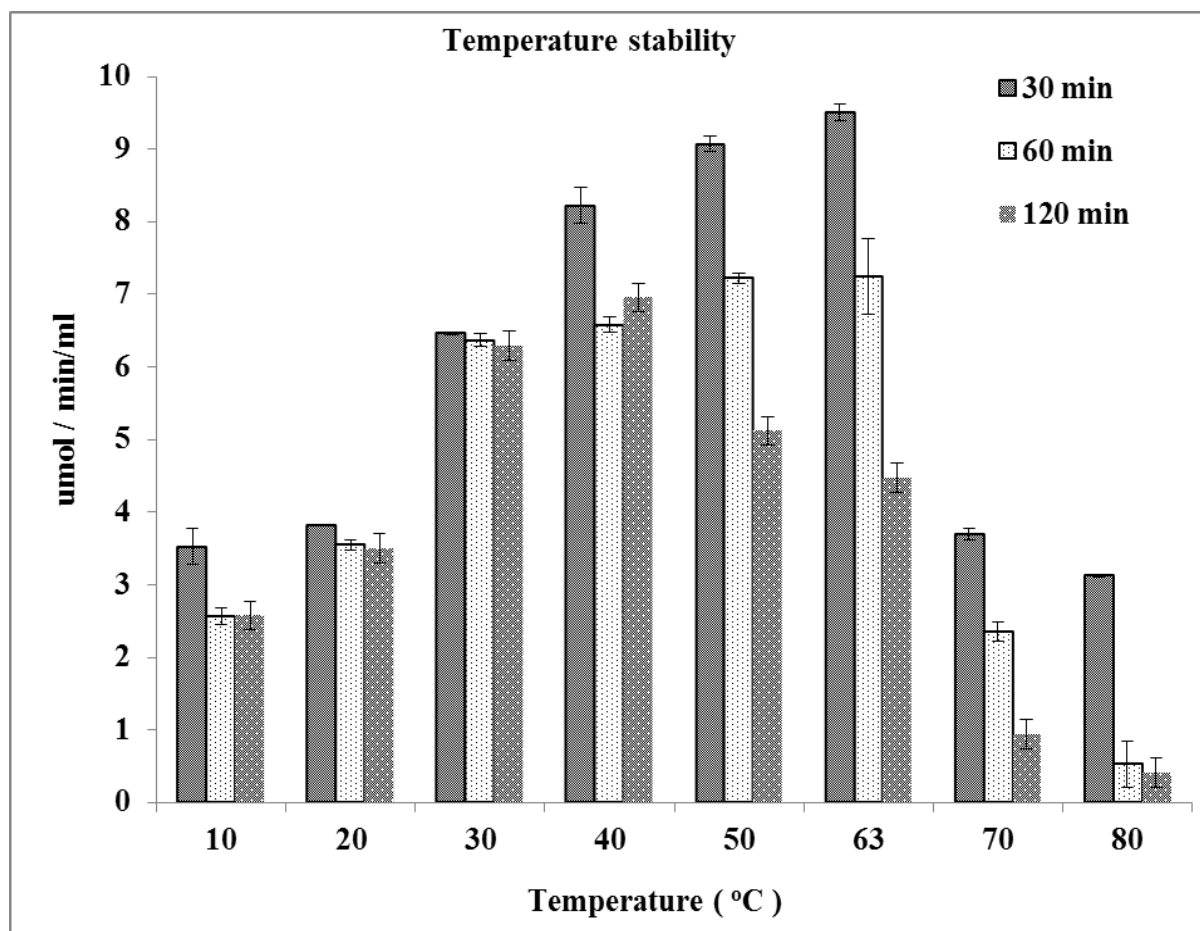


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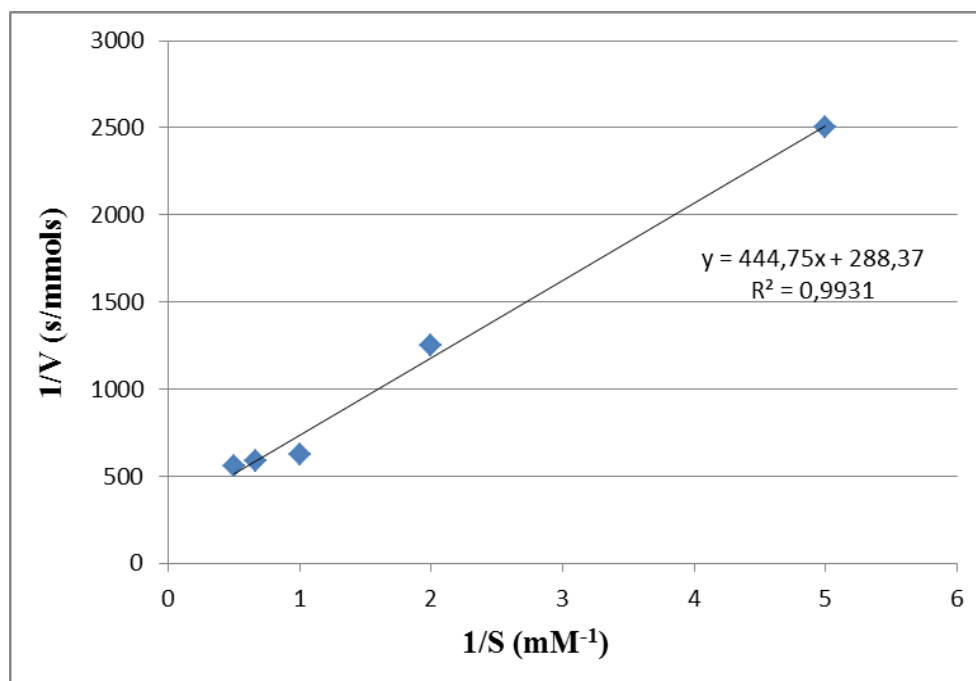


Fig. 5.

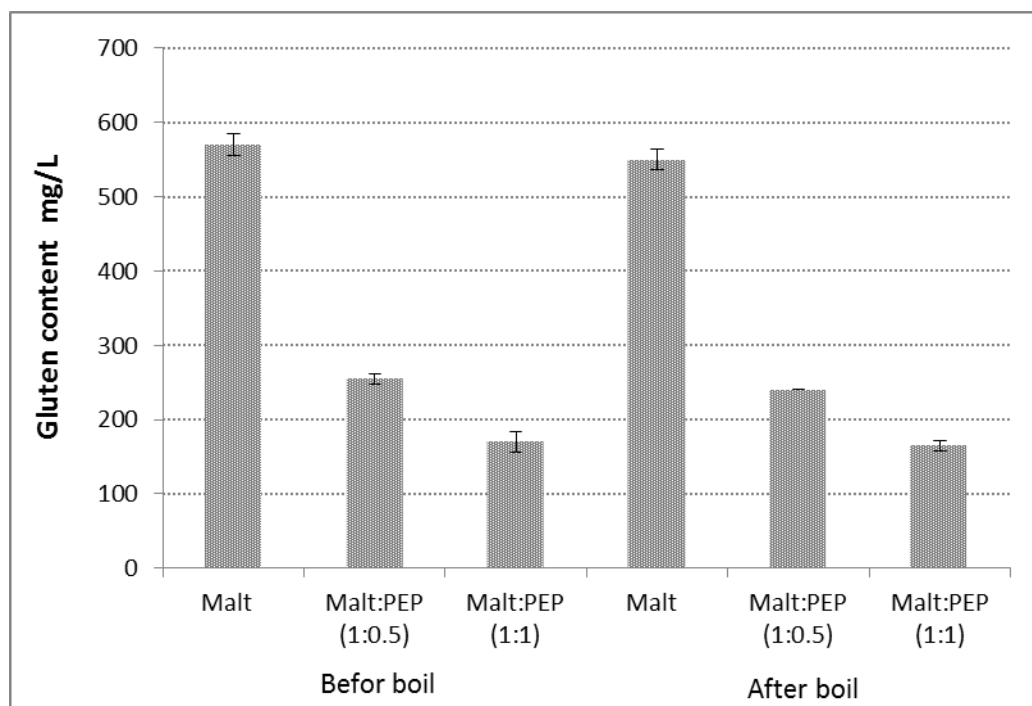


Fig. 6.

Table 1. Effect of various metal ions on the activity of the prolyl endopeptidase from *Sphaerobacter thermophiles*

Metal ions and inhibitors	Concentration	Relative enzyme activity (%)
Enzyme		100.0
Mg ²⁺	1 mM	90.1 ± 7.9
Ca ²⁺	1 mM	83.9 ± 2.1
K ⁺	1 mM	88.8 ± 1.8
Cu ²⁺	1 mM	91.2 ± 5.1
Zn ²⁺	1 mM	91.3 ± 5.2
Na ⁺	1 mM	92.3 ± 2.2
Mn ²⁺	1 mM	90.4 ± 5.3
Co ²⁺	1 mM	102.0 ± 0.4
Fe ³⁺	1 mM	97.3 ± 0.9
Iodoacetate	1 mM	98.3 ± 1.8
PMSF	1 mM	96.0 ± 1.3
EDTA	1 mM	97.9 ± 0.3
EDTA	10 mM	95.3 ± 4.8
EDTA	30 mM	91.2 ± 4.7

Table 2. Peptide fragments identified by MALDI-TOF after incubation with peptides LGQQQPFPPQQPY, PQPQLPYPQPQLPY and SQQQFPQPQQPFPPQQP with PEP enzyme. Where P: Proline; F: Phenylalanine; Q: Glutamine; Y: Tyrosine.

	Amino acid sequence	Preferred cleavage site
Peptide 1	LGQQQPFPPQQPY (α 31–43 gliadin)	LGQQQP FPP QQP Y
Identified fragments	LGQQQP	–/P–F
	LGQQQPFPP	–/P–Q
	FPPQQP	–/P–Y
	FPPQQPY	–/P–F
	QQP	–/P–Q and –/P–Y
	QQPY	–/P–Q
	Y	–/P–Y
Peptide 2	PQPQLPYPQPQLPY (α 62–75 gliadin)	PQPQLPYPQPQLP Y
Identified fragments	PQPQLPYPQPQLP	–/P–Y
	Y	–/P–Y
Peptide 3	SQQQFPQPQQPFPPQQP (γ -hordein)	SQQQFP QPQQP FP QQP
Identified fragments	SQQQFP	–/P–Q
	SQQQFPQPQQP	–/P–F
	SQQQFPQPQQPFPP	–/P–Q
	QPQQPFPPQQP	–/P–Q
	QPQQPFPP	–/P–Q and –/P–Q
	QPQQP	–/P–Q and –/P–F
	FPQQP	–/P–F
	QQP	–/P–Q
	FP	–/P–F and –/P–Q