Heterologous expression of *Hordeum vulgare* protein Z4 in *Pichia pastoris*
shows increased structural stability

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26 **Abstract:** Protein Z4, an abundant of serpin storage proteins of *Hordeum vulgare*, is one major
27 factor that benefits the stability of beer foam. In this study, protein Z4 from Canadian malt
28 Metcalfe was cloned and expressed in *Pichia pastoris*. The glycosylated 45kDa of recombinant
29 protein Z4 was purified by Ni-NTA affinity column. The secondary structure of recombinant
30 protein Z4 was detected via Circular Dichroism (CD) and Fourier Transform Infrared
31 Spectroscopy (FTIR), and β -sheets and β -turns were the main structure of recombinant protein
32 Z4. A few structural differences between recombinant protein Z4 and protein Z4 from malt were
33 also analyzed, that more obvious α -helical structure and more S-H contents were detected in
34 recombinant protein Z4. Meanwhile, higher structural stability of recombinant protein Z4 was
35 also observed after incubating the **samples** in a wide range of temperature, pH and ethanol. These
36 results confirmed the positive relationship between modifications by saccharides and high
37 structural stability of recombinant protein Z4.
38 **Key words:** Protein Z4; *PazI*; *Pichia pastoris*; Secondary structure;

Introduction

Beer is the most consumed alcoholic drink worldwide, and malt is the main raw materials used for brewing [1]. Beer foam, as one important trait of beer, is affected by the chemicals from malt, especially the proteins [2, 3]. In previous researches, proteins were proved to be the most important factors responsible for the quality of beer foam [4]. In beer, there are about 0.5mg/mL of peptides and proteins which are of size 5-100kDa [5]. Most of the proteinaceous materials originate from the cereal material, typically malt. During brewing process, the complex proteolytic system and environment pressures contribute to hydrolyzation and degeneration of soluble proteins. Among them, there are two main albumins including about 9kDa lipid transfer protein (LTP) and 43kDa protein Z, which can resist the enzymatic hydrolysis and survive after the complex environment pressures during brewing process [1]. Previous investigations have found that both protein Z and LTP can influence beer foam. LTP mainly affect the foam formation and protein Z has positive impact on foam stability. The natural LTP in malt has no foaming potential, and modifications during brewing process are the key factors that affect the beer foaming property of LTP [6]. Although foam stability can be enhanced by combined action of LTP and other polypeptides such as protein Z, there is no significant relationship between beer LTP content and the foam stability [7]. Protein Z is the major protein in beer which is one storage protein in the endosperm of malt. Among beer proteins, protein Z is the first found to be beneficial to beer foam for its high surface viscosity and elasticity [4, 8, 9]. Previously, a barley protein Z4 gene *Paz1* was identified and it is suggested that two exons are existed in a protein Z4 consisting 399 residues [10]. On the basis of its high structure stability during brewing process, the existence of protein Z can influence the quality of beer foam [11]. In previous investigations, protein Z can be classified into three isoforms, including Z4, Z7 and Zx [12], however, protein Zx has only been detected in few reports [13]. Among them, protein Z4 accounts for 80% of the total protein Z in malt [7], and the ratio of protein Z4 and Z7 depends on the barley cultivar [14].

It has been reported that protein Z4 and Z7 played positive and negative effects on the stability of beer foam, respectively [15, 16]. The genes encoding Z4 and Z7 are originated from chromosome 4 and 7, respectively [9]. Although most previous reports demonstrated the function of protein Z to beer foam, few reports considered that protein Z4 has no influences on the beer foam stability. Brewing trials were detected using protein Z4-deficient barley as brewing

materials, and protein Z has been indicated to have no greater impacts on foam stability than other beer proteins [17]. In our previous investigations, some structural alterations of protein Z4 were observed during mashing and boiling processes, but the main structure of protein Z4 can still be survived in the extreme environment after brewing process. Protein Z was also monitored to be modified by glycation from the secondary day of malting process [18]. And the modifications of protein Z mainly caused by glycation and glycosylation may be the main reason due to the structural transformations [19]. The modifications have also been considered as one reason for the high endurance of protein Z during brewing process.

The methylotrophic *Pichia pastoris* GS115 has been successfully utilized to express a wide range of heterologous proteins [20], typically eukaryotic proteins. Compared with prokaryotic expression system, this system presents many advantages including high secretion efficiency, high cell density, high production, and easy to scale up. Furthermore, heterologous proteins expressed in *P. pastoris* can also be modified by the protein modification system, such as some degree of glycosylation and hyperglycosylation [21, 22]. In *Pichia pastoris* expression system, the proteins are glycosylated by N-acetylglucosamine and Mannose. Some previous researches demonstrated that modification by saccharides have effects on the secondary structure and biological traits of recombinant proteins, and glycosylation and hyperglycosylation can improve the stability of proteins, especially pH stability and thermal stability [23, 24].

Nowadays, Circular Dichroism (CD) and Fourier Transform Infrared Spectroscopy (FTIR) are considered as the common methods for protein structure analysis. CD measurements provide more accurate information of α -helices in proteins. While FTIR has been used for structural analysis based on the amide groups, especially the amide I region approximately 1700-1600 cm^{-1} [25]. Although the secondary structures of protein Z4 have been explored, the biochemical and structure characterizations have not been fully investigated, especially the effects from glycation and glycosylation on the structural and functional alterations of protein Z4. Meanwhile, the relationships between protein Z4 and the stability of beer foam were still contradictory, and it has been reported that modified protein Z can improve the foam stabilizing properties [26-28]. Therefore, it is necessary to further study on the structural analysis and modifications of protein Z4.

In this work, the cDNA of protein Z4 was cloned via fusion PCR and expressed in *P.*

99 *pastoris* GS115. The secondary structure and structural stability of recombinant protein Z4 in *P.*
100 *pastoris* were analyzed by CD and FTIR. These studies provide the basis for the knowledge of
101 understanding high stability of protein Z4.

102 **Materials and methods**

103 *Strains, Vectors and Culture media*

104 The *PazI* gene of protein Z4 was cloned from Canadian malt Metcalfe purchased from
105 COFCO (Beijing, China). The plasmid pPIC9k and *P. pastoris* host strain GS115 (Mut⁺ his⁻) were
106 from Invitrogen (Carksbad, CA, USA). Mini Kit I and Gel Extraction Kit were obtained from
107 Omega (Georgia, USA). Plasmid pMD19T-simple, *Escherichia coli* JM109, restriction enzymes,
108 T4 DNA ligase, DNA ladder and protein marker were purchased from Takara Co., Ltd. (Dalian,
109 China). Yeast nitrogen base (YNB) without amino acids, G418 Sulfate, DNA primers and DNA
110 sequencing service were from Shanghai Sangon Biological Engineering Technology & Services
111 Co. Ltd. (Shanghai, China). *E. coli* JM109 was grown in LB medium (1% tryptone, 1% NaCl and
112 0.5% yeast extract), *P. pastoris* was cultured in YPD medium (2% peptone, 2% dextrose and 1%
113 yeast extract). Minimal dextrose medium (MD), the buffered minimal methanol-complex
114 medium (BMMY) and the buffered minimal glycerol-complex medium (BMGY) were prepared
115 as described in the Multi-Copy *Pichia* Expression Kit instructions (Invitrogen, Massachusetts,
116 USA).

117 *Cloning, transformation and expression of protein Z4 gene*

118 The exons for protein Z4 were amplified from Canadian malt Metcalfe according to the
119 sequence published at the National Center for Biotechnology Information (NCBI, Accession No.
120 gi|131091) [10]. The two exons were amplified using the following two pairs of primers (Z41-F:
121 tagaattcATGGCAACCACCCTCG; Z41-R:
122 TTGACCAACAGCTTCTAGAGTCTTATGCTGGAAATCCACGGAC and Z42-F:
123 ACCAATCCCCTCATATCTGCATAAGACTCTAGAAGCTGTTGGTCAAG; Z42-R:
124 tgcgggccgcTTAGTGGTGGTGGTGGTGGTGTGCAGATATGAGGGGATT). Restriction
125 enzyme sites *Eco*RI and *Not*I were presented on the underlined bases and the bold His₆-tag was
126 added before terminator codon for purification [29]. The cDNA of protein Z4 gene exclude the
127 intron was cloned by fusion PCR. Fusion PCR was conducted as follows, the system was
128 preheated at 95°C for 10 min, followed by 30 cycles of 95°C for 30s, 63°C for 35s, and 72°C for

90s, and overextended at 72°C for 10min. Then the fusion PCR product was sub-cloned to pMD19T-simple plasmid and transformed into *E. coli* JM109, and then spread on LB plate with ampicillin and cultured at 37°C for 10h. Positive clones were selected and sequenced. After restriction enzyme digestion by *EcoRI* and *NotI*, the excised coding sequence of *PazI* was ligated into pPIC9K by T4 DNA ligase yielding the recombinant plasmid pPIC9K-*PazI* and transformed into *E. coli* JM109, and then spread on LB plate with ampicillin and cultured at 37°C for 10h. After digestion by restriction enzymes *EcoRI* and *NotI* and nucleic acid electrophoresis analysis, the positive recombinant plasmid pPIC9K-*PazI* was linearized with *SalI* and electroporated into *P. pastoris* GS115, pulsing with 2000V for 5ms (Eppendorf Multiporator; Hinz GmbH, Hamburg, Germany). The transformants were spread on MD agar plates and cultured at 30°C for 2-3 days. The positive transformants were detected by colony PCR. And then the selected positive colonies were transformed and selected on YPD plates containing 2.0mg/mL G418. The positive clones which could grow on both MD and YPD/G418 plate were selected for protein expression. The positive recombinants were inoculated into 50mL of BMGY medium and shaken at 30°C at the speed of 180r/min until OD₆₀₀ reached 5-6. Subsequently, all the cells were collected by centrifugation at 4000×g, 4°C for 5min. Then the collected cells were resuspended and inoculated into 200mL of BMMY in a shake flask and cultured at 180r/min, 30°C for 120h. Methanol for induction was added every 24h to the final concentration of 1.0% (v/v) for maintain induction throughout the expression phrase.

Purification and identification of recombinant protein Z4 and protein Z4 from malt

The fermentation broth of recombinant protein Z4 was collected by centrifugation at 8000×g, 4°C for 10min and then the cells were discarded. The supernatant were loaded onto a Ni-NTA affinity column (Qiagen, Wolfenbuttel, German) for purification. The buffers for purification were as follow. Binding buffer: 10mM imidazole/200mM phosphate buffer (pH 6.5). Washing buffer: 20mM imidazole/200mM phosphate buffer (pH 6.5). Elution buffer: 50mM, 100mM imidazole/200mM phosphate buffer (pH 6.5). All the elution solutions were collected and concentrated with a 10kDa molecular weight cut off Pellicon Cassette membrane concentrator (Millipore, MA, USA) for SDS-PAGE detection. The stained single purified protein band from SDS-PAGE was analyzed by MALDI-TOF MS/MS. The band was in-gel digested with trypsin and analyzed via an Ultraflex MALDI-TOF MS/MS spectrometer (Bruker-Daltonics Inc.,

159 Billerica, MA). The spectra were calibrated by the Bruker peptide calibration standard II method.
160 The positive ion reflect mode was selected, meanwhile, Flex Analysis and BioTools softwares
161 were used to analyze the digested peptides. The fragments were identified with an in-house
162 Mascot server on the database of the National Center for Biotechnology Information
163 (<http://www.ncbi.nlm.nih.gov/>) [30, 31]. The protein Z4 from malt was purified as described as
164 previous studies [19].

165 *Deglycosylation of the purified recombinant protein Z4*

166 PNGase F (New England Biolabs, USA) was used to deglycosylate the recombinant protein
167 Z4. According to the guidance, 25 μ L system containing 2 μ L 10 $\times$ Glyco Buffer, 2 μ L 10%NP-40,
168 2 μ L PNGase F, 15 μ g recombinant protein Z4 and 19 μ L sterile water was reacted at 37°C for 20h.
169 After the reaction, the proteins were ultrafiltered with a 10kDa molecular weight cut off Pellicon
170 Cassette membrane concentrator and analyzed by SDS-PAGE.

171 *Circular Dichroism (CD) analysis*

172 The far-UV CD was measured to determine the secondary structure of purified recombinant
173 protein Z4. CD spectra from 190-250nm were collected on a MOS-450/AF-CD
174 spectropolarimeter (BioLogic, France). Sample was dissolved in deionized water to the
175 concentration of 0.1mg/mL. Each spectrum was scanned at a speed of 30nm/min, the response
176 time was 2s, the interval of data points was 1nm and the solvent of protein solution in different
177 reactions was scanned as the background at 25°C [32].

178 *Fourier Transform Infrared Spectroscopy (FTIR) analysis*

179 The FTIR spectra of purified recombinant protein Z4 were recorded via a Thermo Nicolet
180 Nexus 470 FT-IR spectrometer (Thermo Nicolet Analytical Instruments, Massachusetts, USA).
181 Grinding potassium bromide (KBr) and freeze-dried proteins were mixed. The samples were
182 scanned in the range of 4000-400 cm^{-1} . The resolution was 4 cm^{-1} and scanning number was 64
183 times [33]. For the secondary structure analysis of proteins, the amide I region in the FTIR
184 spectrum was also deconvoluted combined with curve fitting.

185 *Effect of Temperature, pH and Ethanol on structural stability of recombinant protein Z4 and* 186 *protein Z4 form malt*

187 The effect of pH on the structural stability of recombinant protein Z4 and protein Z4 from
188 malt were determined over a pH range of 3-11. The pH of solution was adjusted with phosphoric

189 acid solution and sodium hydroxide solution. Samples were dissolved in the solution of different
190 pH to the final concentration of 0.1mg/mL. To investigate the pH structural stability, the purified
191 protein Z4 was incubated in different solution at 20°C for 60min. The structure stability of the
192 treated protein Z4 was measured by CD.

193 For thermal stability determination, the effect of temperature on the protein structure changes
194 was studied via incubating the purified recombinant protein Z4 and protein Z4 from malt in
195 deionized water (concentration were set at 0.1mg/mL for both kinds of protein Z4) as the
196 standard mashing and boiling processes [19]. The mashing process was as follows: First keep the
197 sample at 48°C for 30 min. Then raised the temperature to 63°C and stood still for 40 min, after
198 that raised the temperature to 72°C for 20 min, then raised the temperature to 78°C and finished
199 the simulated mashing process. The rising rate of temperature during each procedure was 1°C
200 /min. Samples were monitored by CD spectrometer at the onset and ending of each temperature
201 procedure during the simulated mashing. After the simulated mashing process, the protein
202 solutions were treated in the boiling water as the boiling process. The samples were detected via
203 CD spectrometer at boiling 30min and the ending of the simulated boiling process.

204 The effects of ethanol on the recombinant protein Z4 and protein Z4 from malt were also
205 tested. Samples were dissolved in ethanol solution to the concentration of 0.1mg/mL. Different
206 concentration of ethanol solution was added and reacted with the purified protein Z4 at 20°C for
207 60min. After reactions, the changes in the secondary structure of protein Z4 as a result of ethanol
208 treatments were determined by CD spectrometer.

209 **Results**

210 *Cloning, expression, purification and identification of protein Z4 in P. pastoris*

211 The two exons of protein Z4 coding sequence were amplified by fusion PCR with two pairs
212 of specific primers. And *PazI* gene was successfully acquired by fusion PCR using the primers
213 Z41-F and Z42-R. The PCR product was then digested with *EcoRI* and *NotI*, and inserted into
214 pMD19T-simple for sequencing. The positive clones were treated with *EcoRI* and *NotI* and
215 inserted into plasmid pPIC9K. The recombinant plasmid pPIC9K-*PazI* was confirmed via
216 enzyme digestion analysis and sequencing. The fusion gene was 1221bp encoding a protein
217 containing 406 amino acids including a His-tag in the carboxy-terminus (Fig. 1). The nucleotide
218 sequence of amplified *PazI* gene was compared with reported *PazI* (*Hordeum vulgare*) gene. As

219 shown in Fig. 1, the cDNA sequence of *PazI* gene from Canadian malt Metcalfe shared 98.5%
220 homology with the reported *PazI* (Accession No. gi|131091, *H. vulgare*) and differed by four
221 nucleotides. Among 50 transformants picked on MD plates, only three colonies grew normally on
222 the YPD/G418 plate. The positive recombinants were screened and named GS115-pPIC9K-*PazI*.
223 After methanol induction in shake flask for 120h, the recombinant proteins in fermentation broth
224 were purified with the Ni-NTA affinity column. The fermentation broth and elution fractions
225 were collected and analyzed by SDS-PAGE. As shown in Fig. 2A, the recombinant protein Z4
226 band was visualized in the fermentation broth. Meanwhile, one purified band (45kDa) was shown
227 in the elution fraction with 100mM imidazole. From the SDS-PAGE results, the His₆-tag was
228 successfully expressed and the Ni-NTA affinity column could be used for purifying recombinant
229 protein Z4. Compared to protein Z4 from malt (43kDa), the increased molecular weight of
230 recombinant protein Z4 might be caused by **glycosylations or hyperglycosylations**. After
231 deglycosylation by PNGase F, the purified band at about 45kDa in the gel almost disappeared.
232 However, a new band was detected which molecular mass was about 43kDa (Fig. 2B). The
233 purified protein was then identified by MALDI-TOF MS/MS and the results showed that the
234 purified band corresponded to protein Z4 (Accession No. gi|131091) (Fig. 3A, Fig. 3B). On the
235 other hand, the calculated molecular weight of protein Z4 from malt was 43kDa, which is lower
236 than that of the estimated untreated recombinant protein Z4. So it can be concluded that the
237 recombinant protein Z4 in the *P. pastoris* was N-glycosylated, and the differences should be
238 caused by **protein modification system in *Pichia pastoris***.

239 *Structural analysis of recombinant protein Z4*

240 The secondary structure of recombinant protein Z4 was measured by CD spectrometer. **As**
241 **showed in Fig. 4**, an obvious negative peak at around 220nm was observed which was one
242 characteristic peak of α -helices caused by the $n-\pi^*$ transformation. Compared with the standard
243 spectrum of α -helices, the spectra of recombinant protein Z4 at around 208nm presented small
244 folding and the absorption at 208nm was not typical (Fig. 4). The results meant that the α -helices
245 of recombinant protein Z4 were not the main structure [34]. The typical positive peak at 195nm
246 demonstrated the existence of β -sheets in the $\pi-\pi^*$ region of protein Z4 from malt [35]. However,
247 the peak was not obvious as shown in the CD spectrum of recombinant protein Z4, where the
248 peaks appeared at about 192nm. These phenomena indicated that recombinant protein Z4

249 obviously presented more α -helical structure than protein Z4 from malt.

250 In order to detect the specific structural differences between recombinant protein Z4 and
251 protein Z4 from malt, FTIR experiments were conducted to analyze the structural characters and
252 functional groups. The FTIR absorptions of recombinant protein Z4 after baseline correction and
253 vectorial normalization were shown in Fig. 5A. There were two strong absorptions presenting in
254 the spectrum at 3405cm^{-1} and 2927cm^{-1} , which contributed to amide A region and amide B region.
255 The two absorption bands were associated with N–H stretching vibrations and C–N stretching
256 vibration of protein, respectively. Compared to the FTIR spectrum of protein Z4 from malt, both
257 of the absorptions peaks moved to high wavenumber, indicating that the N-H stretching
258 vibrations and C-N stretching vibration in recombinant protein Z4 were reinforced. Meanwhile, a
259 strong absorption at around 1080cm^{-1} resulted from C-OH and C-C stretching vibration of
260 saccharides was also observed. A weak absorption peak caused by S-H stretching vibration was
261 also found at around 2429cm^{-1} . To more precisely characterize the recombinant protein Z4, the
262 sensitive amide I region for secondary structural analysis at range of $1700\text{--}1600\text{ cm}^{-1}$ was showed
263 in Fig. 5B. The main peak caused by secondary amide C=O stretching vibration presented at
264 about 1660cm^{-1} and the peak was associated with the helical structure. To reveal more
265 information in the secondary structure of recombinant protein Z4, the amide I region was treated
266 with deconvolution and curve fitting. As shown in Fig. 5C, the original amide I region was
267 separated into 10 peaks as analyzed by Gauss formula combined with deconvoluted and curve
268 fitting. The top spectrum was the original amide I region and the fitting peaks were presented in
269 the bottom of the figure. The quantities of each peak were analyzed and the result was showed in
270 Tab. 1 [36]. All the peaks represented the relative contents of secondary structure of recombinant
271 protein Z4. The total percentage of helices was up to 14.99% in recombinant protein Z4, while
272 the percentage of helices was 13.28% in protein Z4 from malt. This result was identical to the CD
273 result that the recombinant protein Z4 presented relative more helical structure. However,
274 compared to the protein Z4 purified form malt, the relative contents of total β -sheets and β -turns
275 of recombinant protein Z4 were close to protein Z4 from malt after mashing and boiling
276 processes (Supplementary Tab. 1) [19], indicating that they had similar main structure. The ratio
277 of intermolecular β -sheets to intramolecular β -sheets was 0.60, which was the same as that of
278 protein Z4 from wort, indicating that the intramolecular β -sheets had a dominant effect on the

279 β -sheet structure. The results also indicated that parts of the cohesion inside the recombinant
280 protein Z4 were destroyed. The ratio of random coils to helices was 0.94, which was connected
281 with the C=O stretching vibration of peptide bonds. In previous studies, protein Z4 had the
282 structural alterations. Parts of α -helices and β -sheets transformed to β -truns and random coils
283 after mashing and boiling processes, which might be caused by modifications of saccharides.
284 Considering the existences of glycosylations on recombinant protein Z4, these might be one
285 reason for the more relative contents of helical structure than protein Z4 from malt and the
286 similar structure to protein Z4 from wort.

287 *Temperature, pH and ethanol stability of recombinant protein Z4 as determined by Circular*
288 *Dichroism*

289 The effects of the temperature on recombinant protein Z4 and protein Z4 from malt were
290 measured by incubating the reactions in the deionized water as the same procedure of mashing
291 and boiling processes. As shown in Fig. 6A, all the spectra of recombinant protein Z4 during
292 simulated mashing process showed the typical helical negative peak at 220nm. Despite the
293 temperature increased, all the spectra of recombinant protein Z4 remained similar to its original
294 structure and there were no significant absorption shifts being found. During simulated boiling
295 process, slight differences in the CD spectra were observed. Compared with the spectrum of
296 recombinant protein Z4 at the end of mashing process, the spectrum shape of recombinant protein
297 Z4 at the end of boiling process was in slight migration, and the secondary structure of
298 recombinant protein Z4 was minute partially unfolded. However, the main structure of
299 recombinant protein Z4 could still be held after extreme high temperature treatment, and high
300 temperature slightly influenced the recombinant protein Z4. Compared to recombinant protein Z4,
301 the CD spectra of protein Z4 from malt showed more migrations (Fig. 6B), especially during
302 boiling process. The decreased value of ellipticity and slight shift in the positive peak were
303 observed indicating that protein Z4 from malt had more obvious structural transformations which
304 from helical structure to more random coils and the secondary structure of protein Z4 from malt
305 was partially unfolded. Although the influences on protein Z4 from malt from the extreme
306 temperature were more obvious, the structural alterations of both protein Z4 were still relatively
307 minor and the main structure could be maintained. Both of the protein Z4 presented high thermal
308 tolerance.

309 To probe pH structural stability of protein Z4, both recombinant protein Z4 and protein Z4
310 from malt were incubated in the phosphatic solutions from pH 3 to 11 (Fig. 6C & 6D). All the
311 spectra showed the main helical minimum peaks at about 220nm. It was obvious that after being
312 treated, the recombinant protein Z4 was still stable and close to its initial state, where almost all
313 the spectra had the same shape and overlapped which means that the structure of recombinant
314 protein Z4 was stable in different pH buffer. However, the secondary structure of protein Z4 from
315 malt in the same condition had more obvious alterations than recombinant protein Z4 presented,
316 especially when solution pH was lowered to pH 4 and above pH 9. In Fig. 6D, the spectra
317 became bent in the range of 205nm - 220nm and the maximum value moved to the higher
318 wavelength when the pH increased to 11. The transformations presented that a small quantity of
319 α -helices and β -sheets converted to β -turns and random coils. , Protein Z4 from malt was still
320 relatively close to its initial state in a widely range of pH. Meanwhile, the pH environment during
321 brewing process was usually ranging from 4.5 to 5.5, which is more suitable for the structural
322 stability of protein Z4. These observations suggested that the secondary structure of protein Z4
323 from malt was relatively resilient with respect to few substantial changes in the aqueous pH
324 environment.

325 To observe the influence of ethanol on protein Z4, CD analysis was performed following the
326 treatments of protein Z4 with different concentrations of ethanol solutions. There was no
327 variational spectrum observed when the concentration was set below 30%, and no significant
328 peak shifts were revealed (Fig. 6E & 6F). However, when the intensity of ethanol was increased
329 to 70% (v/v), the decrease of ellipticity value of recombinant protein Z4 was observed (Fig. 6E).
330 The spectrum moved towards higher wavenumber, and the ellipticity value had a decreasing
331 tendency, where the contents of β -turns and random coils increased. Despite the secondary
332 structure of protein Z4 was substantially degenerated at high ethanol concentrations, the contents
333 of β -sheets and β -turns which were the main structure of recombinant protein Z4 remained high.
334 However, the significant structural transformations of protein Z4 from malt at 70% ethanol were
335 observed (Fig. 6F). There was a mass of random coils being detected due to the movement of
336 maximum peak to negative value and the minimum peak moved to the lower wavelength. The
337 secondary structure of protein Z4 from malt was destroyed in extreme high ethanol concentration.

338 Discussion

339 In the present study, the *PazI* gene encoding protein Z4 from Metcalfe malt was successfully
340 cloned and expressed in *P. pastoris*. Meanwhile, the recombinant protein Z4 was purified with
341 Ni-NTA affinity column, and the structural stability of extracellular recombinant protein Z4 was
342 measured to be higher than the protein Z4 purified from malt.

343 The recombinant protein Z4 exhibited a molecular mass of 45kDa showed by SDS-PAGE
344 and MOLT-TOF MS/MS results. The molecular mass of the recombinant protein Z4 decreased
345 to about 43kDa after deglycosylated by PNGase F, which was the same as the protein Z4 purified
346 from malt. As it is known, N-glycosylation and hyperglycosylation as the common modification
347 modes occurred to the proteins expressed in *P. pastoris* [37]. Hyperglycosylation was usually
348 found in proteins with 200-300 mannoses modification [38]. In this study, the recombinant
349 protein Z4 was speculated as glycoprotein [39]. From the MALDI-TOF MS/MS and SDS-PAGE
350 results of deglycosylations, the recombinant protein Z4 should be glycosylated by mannose and
351 N-acetylglucosamine. The M8 glycan might be existed, as there were increased peaks of glycan
352 units showing at the aspartic acid residue sites in the mass spectrum results, and the
353 hyperglycosylation of recombinant protein Z4 needs further study. The group alterations in the
354 amino acid residues of recombinant protein Z4 after glycosylated were detected from the related
355 absorption peaks in the FTIR spectrum. Previous studies have proved that the glycosylated
356 expressed protein in *P. pastoris* was essential to keep high thermostability and activity [40]. After
357 deglycosylation, the thermostability of expressed proteins in *P. pastoris* significantly decreased
358 [41, 42]. Therefore, the results suggested that modifications caused N-glycosylations might be an
359 effective reason for higher structural stability of recombinant protein Z4 than protein Z4 from
360 malt presented [23, 43], and the glycosylations could also protect recombinant protein Z4 from
361 enzymolysis [44, 45].

362 It has been proved that modifications on proteins such as glycosylation and glycation could
363 cause remarkable structural changes and physicochemical properties [46-48]. In the researches on
364 another foam related protein lipid transfer proteins (LTP), modifications of LTP can significantly
365 improve the foaming property [42]. In this work, recombinant protein Z4 was glycosylated due to
366 the yeasts' intrinsic glycosylation system. Recombinant protein Z4 shared a similar main
367 structure with the protein Z4 purified from malt, with only a few differences regarding more
368 helical structure in recombinant protein Z4. From the results of FTIR spectrum, more α -helices

369 and less random coils were found in recombinant protein Z4, and the main structure was
370 consisted of β -sheets and β -turns which was similar to protein Z4 from malt after mashing and
371 boiling processes treatment. After modifications by saccharides, recombinant protein Z4 had
372 structural transformation. The modified protein Z4 presented high structural stability to prevent it
373 from degeneration and the final structure showed better ability to stabilize beer foam [19, 20].
374 Although the secondary structure of recombinant protein Z4 after glycosylations was resemble
375 with protein Z4 from malt after brewing process, the influences of thermal, pH and ethanol on
376 glycosylated recombinant protein Z4 was less than the ones on glycosylated protein Z4 from
377 malt.

378 Previous investigations have found that the S-H bonds were beneficial to the extension of
379 the structure, and the extended structure could gather at the air-water interfaces. Here in this
380 research the structure of recombinant protein Z4 was revealed to be more extended. Strong S-H
381 absorption in the FTIR spectrum of recombinant protein Z4 was also obviously observed.
382 Compared to protein Z4 from malt, recombinant protein Z4 had relative more S-H bonds.
383 Meanwhile, the secondary structure of protein Z4 from malt can also be extended after
384 modification during brewing process, and the extended structure had more opportunities to be
385 modified [19, 49]. However, more glycosylations occurred to the recombinant protein Z4 in *P.*
386 *pastoris* might be the main reason causing the structural differences between recombinant protein
387 Z4 and protein Z4 from malt. And most of the modifications should be beneficial to stabilize the
388 structure [19, 49]. It has been reported that modifications on the amidogens of amino acid
389 residues by saccharides could induce the N-H groups in the protein to stretch, and the
390 transformations would cause structural rearrangements and result in structural relaxation. The
391 extended structure made the amphipathic domains and hydrophobic groups of recombinant
392 protein Z4 more easily to attach on the air-water interface of foam and stabilize the beer foam.
393 Therefore, modifications might be the main reason that made the structural transformations of
394 protein Z4. Considering the changes of foam stability after brewing process, the underlying
395 mechanisms of how modifications affecting the structure and ability of protein Z4 to stabilize
396 beer foam still need further investigation.

397 In summary, *PazI* gene was successfully cloned and expressed in *P. pastories*, and the
398 recombinant protein Z4 was more stable in extreme pH, temperature and ethanol environments

399 than protein Z4 from malt. Not only due to its own secondary structure but also the modifications
400 occurred to the recombinant protein Z4 increased the structural stability. Furthermore, these
401 characteristics made recombinant protein Z4 exhibit high structural stability and potential high
402 ability to maintain beer foam.

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536 **Tab. 1** Quantitative estimation of the secondary structure of the recombinant protein Z4 and protein Z4 from malt

Secondary structure	Recombinant protein Z4		Protein Z4 from malt	
	Peak (cm ⁻¹)	Area (%)	Peak (cm ⁻¹)	Aera (%)
Aggregated strands	1614	2.45	1614	1.82
Intermolecular sheets	1625	8.30	1625	14.87
Intramolecular sheets	1635	13.79	1634	18.32
Random coils	1643	14.15	1644	14.58
Helices	1652	14.99	1653	13.28
Turns	1660	14.69	1660	9.71
Turns	1667	11.26	1667	8.66
Turns	1674	10.09	1674	6.10
Turns	1683	5.90	1682	9.98
Turns	1691	4.37	1691	2.72
Total helices	14.99		13.28	
Total sheets	24.54		35.01	
Total turns	46.31		37.17	
Total random coils	14.15		14.58	
Random coils/helices ratio	0.94		1.09	
Intermolecular sheets/Intramolecular sheets ratio	0.60		0.81	

537 **Figure legends**

538 **Fig. 1** The comparison of *PazI* sequences. The reported template sequence of *PazI* was
539 above and the sequencing results was below. The differences of the nucleotide sequences were
540 shown in bold red. The sequence of His-tag was shown in bold.

541 **Fig. 2** Purification and deglycosylation of the recombinant protein Z4. (A) SDS-PAGE of the
542 purifying recombinant protein Z4 by Ni-NTA affinity column. Lane M: Protein marker; Lane 1-2:
543 Fermentation broth of GS115 and GS115-pPIC9K-*PazI*, respectively. Lane 3-4: Flowing through
544 fraction of GS115 and GS115-pPIC9K-*PazI*, respectively. Lane 5-12: Elution fraction with
545 20mM, 50mM, 100mM and 250mM imidazole of GS115 and GS115-pPIC9K-*PazI*, respectively.
546 (B) Lane M: Protein marker; Lane 1: The purified recombinant protein Z4; Lane 2: The
547 recombinant protein Z4 after deglycosylated by PNGase F.

548 **Fig. 3** The identification of the recombinant protein Z4. (A) MOLDI TOF MS/MS spectra of
549 purified protein Z4. Peptide 1: The fragment "LSIAHOTR" circle the peak with 925.533 *m/z*.
550 Peptide 2: The fragment "ELNALAEQVVQFVLANESSTGGPR" circle the peak with 2529.328
551 *m/z*. Peptide 3: The fragment "RLSTEPEFIENHIPK" circle the peak with 1809.975 *m/z*. Peptide
552 4: The fragment "ISYQFEASSLLR" circle the peak with 1413.760 *m/z*. (B) The protein sequence
553 coverage was shown in bold red.

554 **Fig. 4** Circular Dichroism spectrum of the recombinant protein Z4 and protein Z4 from malt.

555 **Fig. 5** Fourier transform infrared spectroscopy analysis of the purified recombinant protein
556 Z4 and protein Z4 from malt. (A) The FTIR spectrum of the recombinant protein Z4 and protein
557 Z4 from malt in the 4000-400 cm^{-1} . (B) The FTIR spectra of amide I region of the recombinant
558 protein Z4 and protein Z4 from malt. (C) Original (top) and curve-fitting (bottom) spectra of
559 amide I region of the recombinant protein Z4 and protein Z4 from malt.

560 **Fig. 6** The effect of temperature, pH and ethanol on the recombinant protein Z4. (A) The
561 purified recombinant protein Z4 was incubated in deionized water as the simulated mashing and
562 boiling process. CD spectrum of samples of the purified recombinant protein Z4 in the water after
563 incubation the reactions as the simulated mashing procedure of 48°C, 63°C, and 72°C ending;
564 63°CStart and 72°CStart: CD spectrum of samples of the purified recombinant protein Z4 in the
565 water after incubation the reactions as the simulated mashing procedure of 63°C and 72°C
566 beginning; Mashing: CD spectrum of samples of the purified recombinant protein Z4 in the water

567 after incubation the reactions after the simulated mashing procedure. Boiling 30min and Wort:
568 CD spectrum of samples of the purified recombinant protein Z4 in the water after incubation the
569 reactions as the simulated boiling procedure of boiling the sample of mashing for 30min and
570 60min, respectively. (B): The purified protein Z4 from malt was incubated in deionized water as
571 the simulated mashing and boiling process. (C) The recombinant protein Z4 was incubated in
572 20mM of phosphatic solutions (pH 3-11). (D) Protein Z4 from malt was incubated in 20mM of
573 phosphatic solutions (pH 3-11). (E) The recombinant protein Z4 was incubated in different
574 concentrations of ethanol. (F) Protein Z4 from malt was incubated in different concentrations of
575 ethanol.

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597 **Fig. 1**

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1  ATGGCAACCAACCTCGCCACCGACGTCGGTCTCTCCATCGCACACCAGACCCGCTTGCC
   |||||||
1  ATGGCAACCAACCTCGCCACCGACGTCGGTCTCTCCATCGCACACCAGACCCGCTTGCC

61  CTCGGCCTCGGTCGCGCATCTCTCCAAACCCGAGCGTGCGCGCAATGCGCTTC
   |||||||
61  CTCGGCCTCGGTCGCGCATCTCTCCAAACCCGAGCGTGCGCGCAATGCGCTTC

121  TCTCCGCTCTCCCTGCACGTCGCACTCAGTCTCATACCGGTGCGCGGCG...GCCACC
   |||||||
121  TCTCCGCTCTCCCTGCACGTCGCACTCAGTCTCATACCGGTGCGCGGCGCGGCGCCACC

178  CGTGACCAGCTCGTCGCCATATCGGGAGATGGGGTGCGAGTGCACGCCAAGGAACGAAC
   |||||||
181  CGTGACCAGCTCGTCGCCATATCGGGAGATGGGGTGCGAGTGCACGCCAAGGAACGAAC

238  GCGCTCGCGGAGCAGGTGGTGCAATTTGTGTCGCCAATGAGTCCAGCAGGCGGCCCG
   |||||||
241  GCGCTCGCGGAGCAGGTGGTGCAATTTGTGTCGCCAATGAGTCCAGCAGGCGGCCCG

298  CGCATCGCCTTCGCCAACGGCATCTTTGTCGATGCGAGTCTCTCGCTCAAGCCTTCCTTC
   |||||||
301  CGCATCGCCTTCGCCAACGGCATCTTTGTCGATGCGAGTCTCTCGCTCAAGCCTTCCTTC

358  GAGGAGCTCGCTGTGTGCCAGTACAAGGCCAAGACACAGTCCGTGGATTCCAGCATAAG
   |||||||
361  GAGGAGCTCGCTGTGTGCCAGTACAAGGCCAAGACACAGTCCGTGGATTCCAGCATAAG

418  ACTCTAGAAGCTGTTGGTCAAGTGAACATCATGGGTAGACCAAGTACCACAGGCTCATC
   |||||||
421  ACTCTAGAAGCTGTTGGTCAAGTGAACATCATGGGTAGACCAAGTACCACAGGCTCATC

478  AAACAGATCCTTCCTCAGGATCTGTTGACAATACTACAAACTAATCCTTGGTAATGCT
   |||||||
481  AAACAGATCCTTCCTCAGGATCTGTTGACAATACTACAAACTGGTCTTGGTAATGCT

538  CTTTATTTCAAAGGGCCTGGGACCAAAAGTTTGATGAATCAAAATACAAATGTGACAGT
   |||||||
541  CTTTATTTCAAAGGGCCTGGGACCAAAAGTTTGATGAATCAAAATACAAATGTGACAGT

598  TTCCACCTTCTTGACGGGAGCTCAATTCAACACAATTCATGTCCAGTACAAGAAGCAA
   |||||||
601  TTCCACCTTCTTGACGGGAGCTCAATTCAACACAATTCATGTCCAGTACAAGAAGCAA

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658  TACATTCTCTCTCCGACAACCTGAAGGTACTTAAGCTTCCTTACGCGAAGGGTCATGAC
   |||||||
661  TACATTCTCTCTCCGACAACCTGAAGGTACTTAAGCTTCCTTACGCGAAGGGTCATGAC

718  AAGAGGCAGTTTTCCATGTACATCTTCTTCTGGAGCACAAGATGGTCTGGAATTG
   |||||||
721  AAGAGGCAGTTTTCCATGTACATCTTCTTCTGGAGCACAAGATGGTCTGGAATTG

778  GCCAAGCGGTTGAGCACTGAACCGGAGTTCATCGAGAACCATAATCCAAAGCAGACGGTT
   |||||||
781  GCCAAGCGGTTGAGCACTGAACCGGAGTTCATCGAGAACCATAATCCAAAGCAGACGGTT

838  GAAGTTGGTCGGTTCAGCTCCCAAGTTCAGATATCATATCAATTGAAGCATCTAGC
   |||||||
841  GAAGTTGGTCGGTTCAGCTCCCAAGTTCAGATATCATATCAATTGAAGCATCTAGC

898  TTGCTCAGAGCTTTGGGTCTCCAATTGCCATTTAGCGAAGAGGCTGATCTGCAGAGATG
   |||||||
901  TTGCTCAGAGCTTTGGGTCTCCAATTGCCATTTAGCGAAGAGGCTGATCTGCAGAGATG

958  GTGGATTCTTCGCAAGGGCTGGAAATCTCACATGCTTCCACAAGTCGTTTGTGAAGTG
   |||||||
961  GTGGATTCTTCGCAAGGGCTGGAAATCTCACATGCTTCCACAAGTCGTTTGTGAAGTG

1018  AACGAAGAAGGAAGTGAAGGCTGGCGCAGCTACAGTTGCAATGGCGTGGCCATGTCAATG
   |||||||
1021  AACGAAGAAGGAAGTGAAGGCTGGCGCAGCTACAGTTGCAATGGCGTGGCCATGTCAATG

1078  CCCCTGAAGGTGGATTGGTGGATTGTGTCGGAATCAACCTTTCTCTTCTTATTCGG
   |||||||
1081  CCCCTGAAGGTGGATTGGTGGATTGTGTCGGAATCAACCTTTCTCTTCTTATTCGG

1138  GAAGACATTGCCGGTGTGGTGGTCTTCGTAGGTGATGTGACCAATCCCTCATATCTGCA
   |||||||
1141  GAAGACATTGCCGGTGTGGTGGTCTTCGTAGGTGATGTGACCAATCCCTCATATCTGCA

1198  TAA.....

1201  GTGGTGGTGGTGGTGGTAA

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605 **Fig. 2**

629 **Fig. 3**

(A)

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1  MATTLATDVR LSIAHQTRFA LRLRSAISSN PERAAGNVA F SPLSLHVALS
51 LITAGAAATR DQLVAILGDG GAGDAKEELNA LAEQVVQFVL ANESSTGGPR
101 IAFANGIFVD ASLSLKPSFE ELAVCQYKAK TQSVDFQHK T LEAVGQVNSW
151 VEQVTTGLIK QILPPGSVDN TTKLILGNAL YFKGAWDQK F DESNTKCDSF
201 HLLDGSSIQT QFMSSTKKQY ISSSDNLKVL KLPYAKGHD K RQFSMYILLP
251 GAQDGLWSLA KRLSTEPEFI ENHIPKQIVE VGRFQLPKF K ISYQFEASSL
301 LRALGLQLPF SEEADLSEMV DSSQGLEISH VFHKSFVEV N EEGTEAGAAT
351 VAMGVAMSMP LKVDLVDFVA NHPFLFLIRE DIAGVVVFVG HVTNPLISA

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(B)

640 **Fig. 4**

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659 **Fig. 5**

(C)

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663 **Fig. 6**