The Formation and Hydrolysis of Barley Malt Gel-Protein Under Different Mashing Conditions

S. Pöyri,[1,4] M. Mikola,[2,3] T. Sontag-Strohm,[2] A. Kaukovirta-Norja,[1] and S. Home[1]

ABSTRACT

J. Inst. Brew. 108(2), 261–267, 2002

The effect of oxidation and proteolysis on the amount of gel-protein aggregate was investigated both *in vivo* during mashing and *in vitro*. The oxidation of the free thiol groups of proteins to disulphide bridges during mashing appeared to be a good indicator of the formation of gel-protein aggregate. The pH optimum of the oxidation varied according to the isothermal mashing temperature. The results suggested that the oxidation of the thiol groups maybe a result of some kind of enzymatic activity. In vitro experiments showed that the proteolysis of the gel-protein aggregate was strongest at pH 5.0 and temperature denaturation occurred only at temperatures over 80°C. Mashing experiments on the other hand suggested that the proteolysis of the monomer subunits of gel-protein (i.e. B- and D-hordein) had a stronger effect on the final amount of the gel-protein aggregate than the hydrolysis of the aggregate.

Key words: Gel-protein, hordein, hydrolysis, mashing, oxidation, proteolysis.

INTRODUCTION

The barley gel-protein is a polymer that is formed by polymerisation of high molecular weight (HMW; ca. 95 kDa) and low molecular weight (LMW; ca. 40 kDa) subunits which are considered to be barley storage proteins, called D hordein and B hordein respectively[26,27,30]. Moonen et al.[19] have hypothesized that in barley HMW subunits form a backbone, which binds LMW subunits with disulphide bridges under oxidative conditions to form a gel-like aggregate. The reducing conditions cause disruption of disulphide bridges diminishing the amount of gel-protein aggregate during malting but in mashing oxygen is present and the aggregate is reformed[20]. The gel-protein of malt represents a major part of the so called Oberteig-layer that is formed on top of the spent grains during lautering and is known to retard wort separation[21]. Moreover, wort separation can be accelerated by adding reducing substrates at mashing that disrupt the disulphide bridges[29]. Recent results[24] have shown that oxidation during mashing has an effect on the amount of gel-protein in spent grains and on the wort separation rate.

Through the malting and mashing processes barley proteins are also hydrolysed by proteolytic enzymes. During germination of barley the hordeins are partially degraded with D hordein being attacked first and then B and C hordeins[32]. The D hordein is also degraded more rapidly than B and C hordeins when subjected to hydrolysis in vitro by a purified 30 kDa cysteine proteinase[23]. Less proteolysis occurs during mashing than in the malting process but still slightly less than a quarter of the soluble nitrogen containing substances of wort are formed at this point of the process by the endoproteinases[13].

The aim of this work was to study the net results of formation and hydrolysis of gel-protein during mashing and possibilities for preventing its formation.

The effects of oxidation level, temperature and pH on the amount of gel-protein were studied. In addition an attempt was made to characterize the malt endoproteinases that may be responsible for gel-protein hydrolysis during mashing.

MATERIALS AND METHODS

Mashing trials

Commercial Kymppi malt harvested in 1996 was used for the experiments. High-gravity mashings for preparing high gravity worts were carried out according to a mashing procedure (HGM) mimicking brewery practice (malt: water ratio 1:4, temperature programme 48°C 30 min /63°C 30 min/ 72°C 30 min / 80°C 10 min). The temperature increase rate was about 1.5-2°C/min[25]. The pH was adjusted with 0.5 M H_2SO_4 (0-18 mL/L) to give mash pHs of 6.0 to 4.6 (measured at 20°C). At mashing temperatures the pH is somewhat lower than in the cooled wort[12]. After mashing wort was separated in a Büchner-funnel and spent grains were collected and freeze-dried.

Isothermal mashings were done at temperatures of 40, 50, 60, 70, 80 and 90°C for 60 min. After mashing, sam-

TABLE I. The effect of added H_2SO_4 on the achieved pH of the cold worts after isothermal mashings at 40-90°C.

Addition of H_2SO_4 (mL/L)	Range of achieved pH values
0	5.9-5.7
1.5	5.8-5.6
8	5.4-5.1
11	5.2-4.9
15	4.9-4.7
18	4.7-4.6

[1] VTT Biotechnology, PO Box 1500, FIN-02044 VTT, Finland
[2] University of Helsinki, Department of Food Technology, PO Box 27, FIN-00014 University of Helsinki, Finland
[3] Current address, AVENA oy, Kansakoulunkatu 1 B, FIN-00100 Helsinki, Finland
[4] Corresponding author. E-mail: saara.poyri@vtt.fi

Publication no. G-2002-0612-03R
© 2002 The Institute & Guild of Brewing

ples were immediately cooled (<5°C) in ice-water and centrifuged (8 000*g*, 10 min, +4°C). Unwashed spent grains were freeze-dried.

Isolation of gel-protein

Gel-protein was isolated from freeze-dried spent grains or barley. The material was milled through a 0.5 mm sieve (Fritsch GmbH). The flour (4g/100 mL) was extracted with petroleum ether (B.P. 40-60) for an h to remove excess of lipids. Gel-protein was isolated by extracting flour with 63 mL of cold 1.5% SDS (sodium dodecyl sulphate) for 10 min and centrifuging the material in a Beckman Ultracentrifuge (70 000g) for 40 min[11]. Gel-protein is insoluble in SDS and it forms a gel-like material on the top of the flour pellet. The gel was carefully scraped off the pellet, solubilised in 8M urea with 5% 2-mercaptoethanol and the amount of the soluble protein was measured using the Bradford method[5].

Degree of oxidation

Degree of oxidation (DoO) was measured as a ratio of free thiol groups and disulphide bridges in wort during mashing.

Free thiol group content

The amount of free thiol groups of the wort was measured with the colorimetric method first described by Ellman[9,10]. Wort (200 µL) was incubated for 30 min with 1 mL of reaction buffer containing 0.4 mM 5,5'-dithiobis 2-nitrobenzoic acid (DTNB; Sigma), 0.2 M Tris-HCl, pH 8.0, 1% SDS and 3mM EDTA. The absorbance of the solution was read at 412 nm. Blank samples of wort with buffer without added DTNB were used as a reference. The method was calibrated with cysteine hydrochloride monohydrate (Merck).

Total sulphydryl group content

The amount of disulphide groups was calculated as the difference between sulphydryl groups before and after reduction of disulphide bridges with sodium sulphite.

The amount of free thiol groups after reduction was measured with NTSB (2-nitro-5-thiosulphobenzoate)[6,28]. The amount of 200 µl of diluted wort (1:10 with water) was incubated for 30 min with 1 mL of reaction buffer consisting of 1mM NTSB, 0.1M sodium sulphite, 0.2 M Tris-HCl, pH 9.5, 1% SDS and 3mM EDTA. NTSB was synthesized from DTNB in the presence of sodium sulphite and O_2 as described by Thannhauser[28]. The absorbance of the solution was read at 412 nm. A blank sample of wort and buffer without added NTSB was used as a reference. The method was calibrated with cysteine hydrochloride monohydrate (Merck).

Calculation of DoO

$$\mathrm{DoO}(\%) = 100 - \frac{[SH_{\mathrm{free}}]*100}{2*[S-S]+[SH_{\mathrm{free}}]}$$

$[SH_{\mathrm{free}}]$ = free thiol group content

$[S-S]$ = the amount of disulphide bridges (total sulphydryl group content – free thiol group content)

In order to measure the degree of oxidation during mashing the results were compared with the blank malt extract. Malt was extracted with deaerated water (1:4 malt: water ratio) for 10 min at room temperature under a nitrogen blanket to minimize oxidation.

$$\mathrm{Relative\ DoO}(\%) = \frac{(\mathrm{DoO} - \mathrm{DoO}_0)*100}{100 - \mathrm{DoO}_0}$$

DoO = Degree of oxidation of a sample

DoO_0 = Degree of oxidation of a blank malt extract

In vitro hydrolysis of barley gel-protein by malt endoproteinases

The endoproteinases were extracted from barley malt according to Zhang and Jones[33]. One gram of malt was homogenized with 2 mL of 50 mM Na-acetate buffer (pH 5.0) including 2 mM cysteine. Extraction was carried out under agitation at + 4°C for one h followed by centrifugation (10,000g, 4°C, 15 min) after which the supernatant was frozen in aliquots and used for hydrolysis experiments.

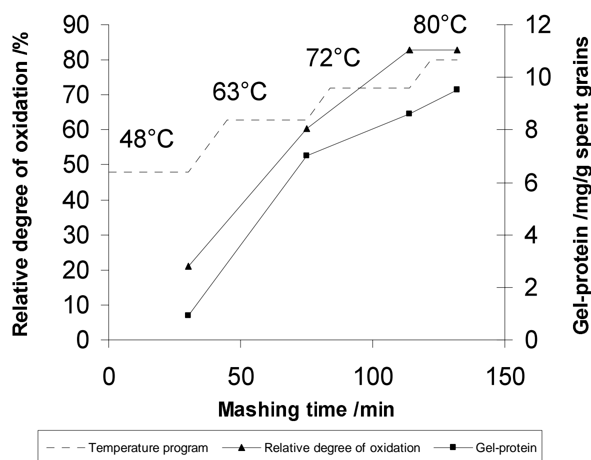

FIG. 1. Changes in the degree of oxidation and the amount of gel-proteins in spent grains during mashing. Results are representative data from series of experiments (n>30).

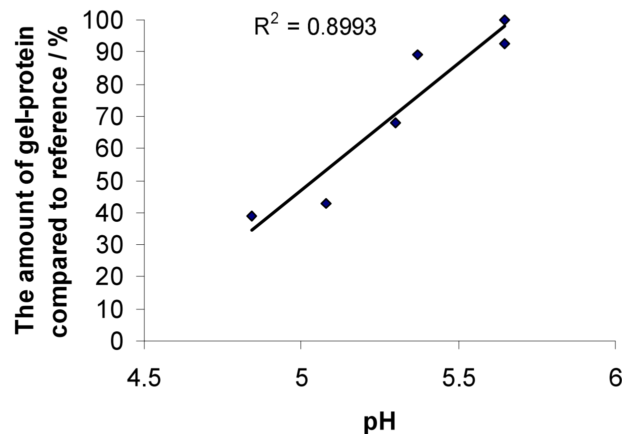

FIG. 2. The effect of mashing pH on the amount of gel-proteins in spent grains compared to the HGM procedure with a pH of 5.6. Results are from series of mashings made by using statistical design.

For the model hydrolysis experiments 0.25 mL of 1% (w/v) of freeze dried gel-protein preparation was mixed with 0.5 mL of appropriate buffer including possible additives and then the reaction was started by adding 0.25 mL of endoproteinase extract. Hydrolysis was carried out at 40°C and the reaction was stopped by adding reducing SDS sample buffer and incubating the mixture for 5 min in a boiling water bath.

SDS-PAGE on 12% homogeneous gels was performed and the buffers were prepared according to the manufacturers instructions (MiniProteanII, BioRad Laboratories, Hercules, CA). After the separation of proteins the gels were stained using 0.06% Coomassie brilliant blue R-250 in 6% trichloracetic acid overnight. The gels were destained in water until the backgrounds were clear and photographed.

RESULTS AND DISCUSSION

Formation of gel-protein aggregate during mashing

In the present study the formation of aggregate (gel-protein) in the high gravity mashing (HGM) was approximately parallel to the relative degree of oxidation measured as the oxidation of free thiol groups (Fig. 1). The formation of gel-protein was quite slow and the oxidation minimal at the temperature of the first rest (48°C) but during and after the saccharification rest (63°C) the mash was oxidized vigorously and the amount of gel-protein in spent grains rose rapidly. At the temperatures over 72°C the oxidation ceased but the amount of gel-proteins kept increasing.

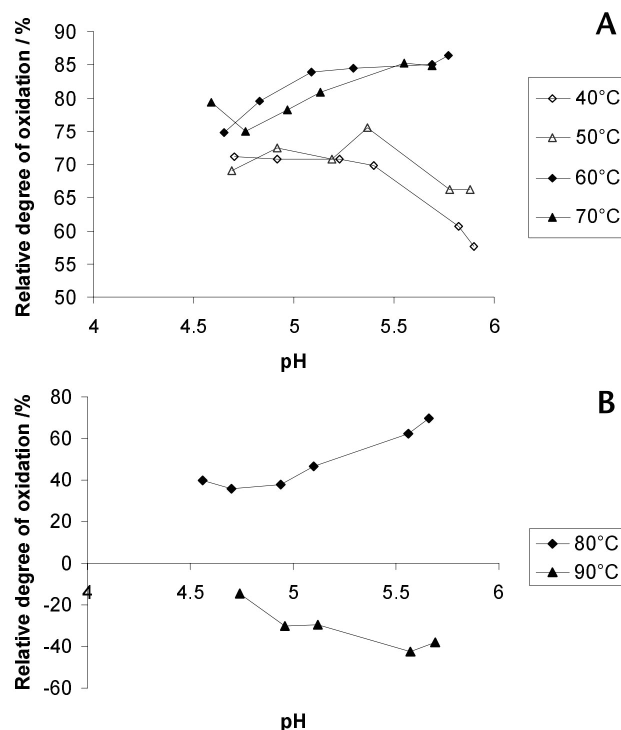

FIG. 3. The effect of pH on the degree of oxidation in isothermal mashings at temperatures of 40-70°C (A) and 80-90°C (B). Results are averages of triple measurements.

Effect of mashing conditions on the amount of gel-protein

Oxidation-reduction reactions are known to be influenced by pH. In order to find out what effect a mash pH has on the formation of gel-protein a series of high-gravity mashings were conducted under different pH conditions. The HGM procedure at pH 5.6 was used as a reference. The amount of gel-protein in spent grains decreased approximately linearly with mash pH-value (Fig. 2). At pH 4.8 a decrease of 60% in gel-protein content of spent grains was measured compared to the reference mashing. This decrease in gel-protein did not accelerate the wort separation rate (results not shown). Probably the starch that remains unhydrolysed at low pH conditions has a stronger adverse effect on wort separation. The decrease of gel-protein at low pH indicates that endoproteinase activity may have an impact on the formation of gel. Cysteine endoproteinases (the major class of proteinases in

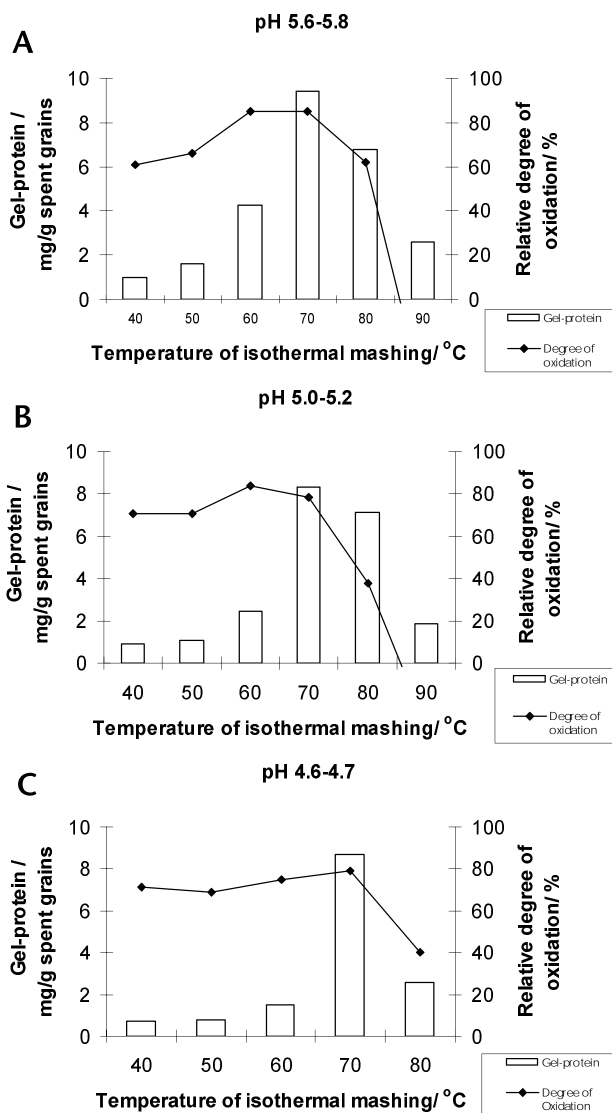

Fig. 4. The amount of gel-proteins in spent grains after isothermal mashings at different temperatures with addition of 1.5 (A), 11 (B), and 18 (C) mL/L of sulphuric acid.

malt) have their pH optima near 3.8-4.5[33]. Thiol-oxidation is also pH dependant but as can be seen from Figures 4 A-C the difference in oxidation with different pH values is much smaller than the difference in formation of gel-proteins (Fig. 2).

Moreover, the effect of pH and temperature together on the oxidation of mash and on the formation of gel-protein was studied in isothermal mashings at six different temperatures (40-90°C) and with addition of six different amounts of 0.5 M H_2SO_4 (0, 1.5, 8, 11, 15 and 18 mL/L). The achieved pH values of the cold worts varied depending on mashing temperature (Table I).

The relative degree of oxidation of mash could be divided into three categories according to mashing temperatures (Fig. 3A). Oxidation in isothermal mashings (60 min) at temperatures of 40°C and 50°C occurred at a medium rate and was enhanced at low pH levels. At temperatures of 60°C and 70°C oxidation was more rapid than at lower temperatures but the degree of oxidation was lower when pH decreased. At a temperature of 80°C the DoO was small when the pH was low (Fig. 3B). At 90°C the conditions caused reducing of disulphide bridges at every pH value. In mashing at 90°C with acid addition of 18 mL/L (pH under 4.6) the separation of wort from solid particles was so difficult that no analysis could be carried out. This was probably due to negligible starch hydrolysis.

Present results suggest that the formation of gel-protein is, at least partly, related to oxidative enzyme activity dur-

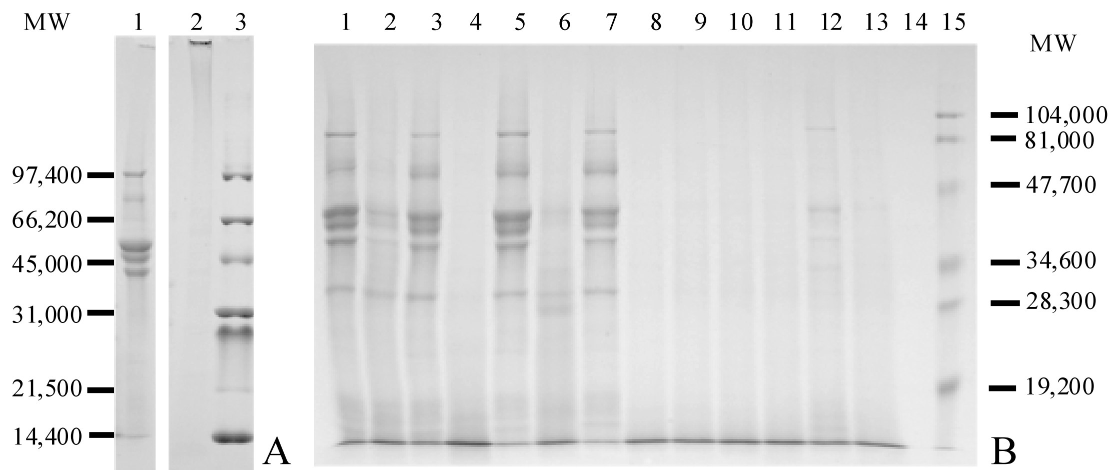

FIG. 5. The effect of pH and class specific proteinase inhibitors on model digestion of barley gel-proteins by malt endoproteinases. Hydrolysis products analysed by 12% homogenous SDS-PAGE. A, lane 1, gel-protein preparation, reduced; lane 2, gel-protein preparation, nonreduced; lane 3, molecular weight standard. B, lane 1, pH 3.8, 0 h; lane 2, pH 3.8, 24 h; lane 3, pH 5.0, 0 h; lane 4, pH 5.0, 24 h; lane 5, pH 6.2, 0 h; lane 6, pH 6.2, 24 h; lanes 7-13, pH 5.0; lane 7, as lane 3; lane 8, as lane 4; lane 9, as lane 8, methanol added; lane 10, as lane 8, o-phen added; lane 11, as lane 8, pep A added; lane 12, as lane 8, E-64 added; lane 13, as lane 8, PMSF added; lane 14, empty; lane 15, molecular weight standard.

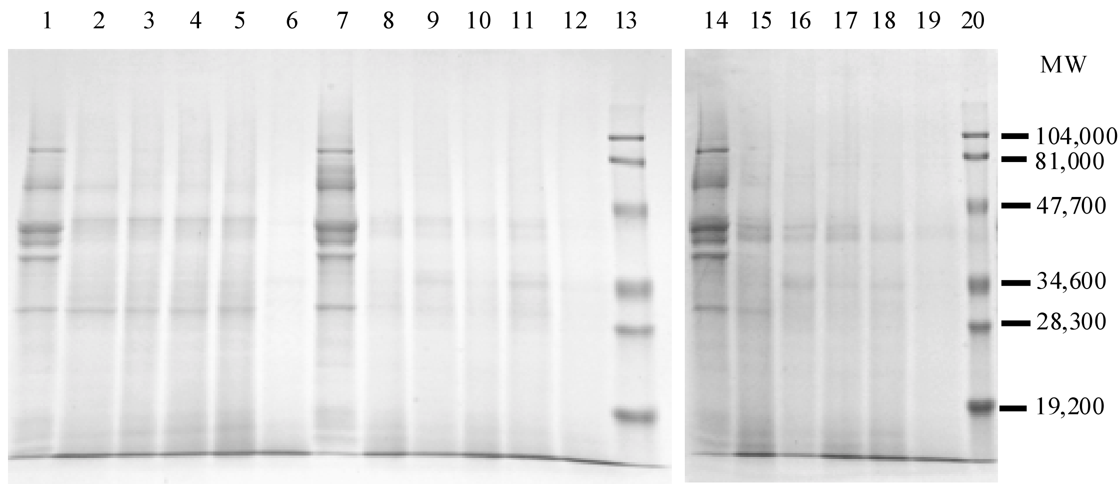

FIG. 6. The effect of a narrow pH range on model digestion of barley gel-proteins by malt endoproteinases. The conditions were as described in Materials and Methods and the hydrolysis products were analysed by 12% homogenous SDS-PAGE. Lanes 1-6, pH 4.5; lanes 7-12, pH 5.0; and lanes 14-19, pH 5.5. Lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 3 h; lane 5, 4 h; lane 6, 24 h; lane 7, 0 h; lane 8, 1 h; lane 9, 2 h; lane 10, 3 h; lane 11, 4 h; lane 12, 24 h; lane 13, molecular weight standard; lane 14, 0 h; lane 15, 1 h; lane 16, 2 h; lane 17, 3 h; lane 18, 4 h; lane 19, 24 h; lane 20, molecular weight standard.

ing mashing. Most oxygen-scavenging enzymes are able to catalyse the formation of disulphide bridges. The most common oxygen scavenging enzymes that are active in barley malt are peroxidase, catalase, superoxidedismutase and lipoxygenase.

Catalase and superoxidedismutase are known to be inactivated rapidly during mashing at 65°C[1,7]. Peroxidase can retain its activity even at 80°C[3,7]. Peroxidases are considered to act mostly on the oxidation of polyphenols. In the present experiments the mashing pH and temperature did not have the same impact on oxidation of polyphenols as on oxidation of disulphide bridges (results not shown). It seems that the formation of disulphide bridges during mashing is mostly related to some other enzyme.

Kaukovirta-Norja[14] found a correlation between lipoxygenase activity of malt and the wort separation. This effect may also be related to oxidation of free thiol groups of gel-protein[17]. The function of lipoxygenase (LOX) during mashing is rather controversial[2,16]. Lipoxygenase is suggested to be heat labile because only a minor part of the activity present in green malt survives kilning. However, this remaining part of LOX is known to be very stable towards heating. According to Baxter[4] almost 60% of the LOX activity of malt extract survived for 1 h at a temperature of 67°C. Recent results also indicate that LOX may survive temperatures as high as 95°C in spent grains[31]. In addition, Doderer[8] reported that lipoxygenase purified from germinating barley has a pH optimum at 6.5. This is in agreement with the present results where oxidation increased as the pH increased from 4.5 to 6. The effect of pH higher than 6 has not been studied in this experiment.

The enhancement of proteolysis by low pHs and its possible effect on the amount of gel-protein aggregate can be seen by comparing Figs. 1 and 4 A, B and C. In the HGM procedure after the first 30 min at 48°C the relative degree of oxidation was ca 20% and the amount of gel-protein aggregate was ca. 1 mg / g spent grains. In isothermal mashings after 1 h at 50°C the degree of oxidation was over 65% but the amount of aggregate was still 1 mg/g. It seems that the proteases cleaved either gel-protein subunits or the aggregate. The same phenomena could be seen with the HGM procedure at 63°C and the isothermal mashing at 60°C. At these temperatures degradation of gel-protein could be enhanced by lowering the pH (Fig. 4 A, B, C). In isothermal mashings there was a dramatic increase in the amount of aggregate when the temperature was raised from 60 to 70°C (Fig. 4A,B, C). At the mashing temperatures of 72°C in HGM and 70°C in isothermal mashings the relative degree of oxidation was ca. 80% and the amount of gel-protein was near its maximum at 8-9 mg/g. No effect of proteolysis could be seen. At temperatures over 80°C there was less oxidation and the formation of gel-protein aggregate was minor. Although proteolysis at such a high temperature is negligible, denaturation and precipitation of proteins also diminishes the amount of aggregate.

Model experiments of gel-protein hydrolysis

The final amount of the gel-protein aggregate after mashing depends highly on the balance between formation and hydrolysis of gel-protein. In order to find out the real impact of protein hydrolysis on the amount of gel-protein a series of model experiments was made with barley gel-protein. Barley gel-protein was chosen for the substrate because in the reducing conditions during malting the amount of gel-material diminishes and purification of the protein is quite difficult.

The gel-protein fraction of barley consisted mainly of proteins with molecular weights of 98 kDa and 40-55 kDa (Fig. 5A lane 1). According to Smith[26,27] these are D and B hordeins. When this fraction was electrophoresed under non-reducing conditions none of the protein could penetrate into the gel indicating a polymeric nature (Fig. 5A lane 2). The protein band between 28.3 kD and 34.6 kD

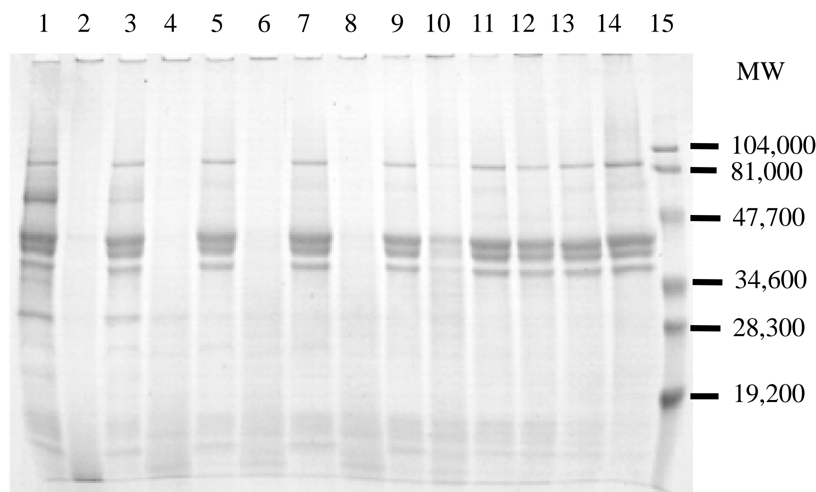

FIG. 7. The effect of heat treatment on model digestion of barley gel-proteins at pH 5.0 by malt endoproteinases. Prior to the hydrolysis reaction, the enzyme extracts were pre-incubated for 15 min at temperatures shown below. The reaction conditions were as described in Materials and Methods and the hydrolysis products were analysed by 12% homogenous SDS-PAGE. Lane 1, no treatment, 0 h; lane 2, no treatment, 24 h; lane 3, 60°C, 0 h; lane 4, 60°C, 24 h; lane 5, 65°C, 0 h; lane 6, 65°C, 24 h; lane 7, 70°C, 0 h; lane 8, 70°C, 24 h; lane 9, 80°C, 0 h; lane 10, 80°C, 24 h; lane 11, 90°C, 0 h; lane 12, 90°C, 24 h; lane 13, 100°C, 0 h; lane 14, 100°C, 24 h; lane 15, molecular weight standards.

molecular weight markers (for example lane 1gel 5B) which is visible in Figs. 5-7 is not derived from the substrate gel-protein but from the enzyme extract. The gel-protein preparation was incubated at 40°C for 24 h with proteinase mixture extracted from malt. These hydrolysis reactions were carried out at three different pH values (pH 3.8, 5.0 and 6.2). Different patterns of hydrolysis were detected (Fig. 5). The D hordein was hydrolyzed at all of the pH values (Fig. 5B lanes 1-6). At pH 3.8 some of the B hordein was not hydrolysed (Fig. 5B lanes 1,2). This was also the case at pH 6.2 although not as clearly as at pH 3.8 (Fig. 5B lanes 5,6). At pH 5.0 no proteins after the incubation were detectable (Fig. 5B lanes 3,4).

In order to find out the nature of the proteinases participating in the hydrolysis of the gel-protein the class-specific proteinase inhibitors were added into the reaction mixtures at pH 5.0. Only minor effects were detected (Fig. 5B lanes 7-14). O-phenanthroline (metalloproteinase inhibitor, Fig. 5B, lane10), PMSF (serine proteinase inhibitor, lane 13) and pepstatin A (aspartic proteinase inhibitor, lane 11) had no effect on the hydrolysis products. However, E-64 a cysteine proteinase inhibitor partially inhibited the hydrolysis (lane 12) suggesting a role of cysteine proteinases in the reaction. This is in agreement with earlier results showing that cysteine proteinases most probably are responsible for the bulk hydrolysis of the hordeins during barley germination[15,22,23].

To narrow the area of the pH optimum further, tests were then conducted using shorter incubation periods; samples were taken at 1h intervals up to 4 h at pH 4.5, 5.0 and 5.5 (Fig. 6). The hydrolysis proceeded fastest at pH 5.0 (Fig. 6 lanes 7-12), slower at 4.5 (Fig. 6, lanes 1-6) and most slowly at pH 5.5 (Fig. 6 lanes 14-19). Further analyses were performed at pH 5.0 since it was the optimum pH for the gel-protein hydrolysis and this is also within the area of pH 4.9-5.1, which has been reported to be the pH of germinating barley endosperm[18].

To study the thermal stability of the proteinases the enzyme extract was pre-incubated for fifteen min at different temperatures prior to hydrolysis reactions (Fig. 7). Heating at 50, 60, 65 or 70°C did not alter the extent of hydrolysis (Fig. 7 lanes 1-8). Pre-incubation at 80°C did affect the hydrolysis, because all of the substrate-derived protein bands were visible after the hydrolysis but the intensity of the bands was lower than in the beginning (Fig. 7 lanes 9,10). Pre-incubation at 90 or 100°C inactivated the proteinases totally so that the substrate polypeptides were intact after the incubation (Fig. 7 lanes 11-14).

Addition of an oxidizing agent, hydrogen peroxide, partially inhibited the hydrolysis (results not shown) which would be logical since the catalytic activity of cysteine proteinases requires that their active site cysteines are reduced.

CONCLUSIONS

The oxidation of the mash was determined with a Degree of Oxidation (DoO) method based on the oxidation of free thiol groups to disulphide bridges in mash. The method gave also a good impression of the formation of gel-protein aggregate during mashing.

The Degree of Oxidation during mashing was dependent on the pH and the temperature of the mashing. The different pH optima of the oxidation in different temperatures implied that the oxidation of the thiol groups to disulphide bridges maybe a result of enzymatic activity, possibly lipoxygenase activity.

Gel-protein hydrolysis was found to have a clear pH optimum near pH 5.0 according to model experiments. The same result was not observed in mashing experiments. Instead the amount of gel-protein decreased further when the pH was lowered under 5.0. Since the proteolysis of gel-protein subunits (D- and B-hordeins) by cysteine proteinases has a pH optima near 3.8-4.5 it seems that the proteolysis of D- and B-hordeins has a stronger effect on the hydrolysis-formation -balance of the gel-protein during mashing than the proteolysis of gel-protein aggregate (pH optimum at 5.0). Also the oxidizing conditions during mashing may have an effect on the proteolytic activity of cysteine endoproteinases and alter their characteristics.

There was also a difference between the thermal inactivation of enzymes that hydrolyse gel-protein. In model experiments thermal inactivation happened only at temperatures over 80°C while in mashing experiments no proteolysis could be observed after 70°C. It is possible that the extraction of the malt enzymes in model experiments may offer some protection against thermal inactivation.

As a conclusion the balance between formation and hydrolysis of gel-protein aggregate appears to be difficult to control. It is not possible to adjust the pH value low enough to accelerate gel-protein hydrolysis without causing inefficient starch hydrolysis. Lengthening the time of the proteolysis rest is also not suitable because it causes formation of excess FAN. Instead, the level of oxygen can be adjusted without adverse effects. The amount of the gel-protein aggregate can be diminished by preventing oxidation and furthermore the wort separation is improved.

REFERENCES

1. Bamforth, C.W., J*ournal of the Institute of Brewing*, 1983, **89**, 420.
2. Bamforth, C.W., *Journal of the Institute of Brewing*, 1999, **105**, 237.
3. Bamforth, C.W., Hughes, P.S., Muller, R.E., Walters, M.T., Antrobus, C.J. and Large, P.J., Proceedings of the 6th International Brewing Conference, Harrogate, 1996, p. 267.
4. Baxter, D., *Journal of the Institute of Brewing*, 1982, **88**, 390.
5. Bradford, M.M., *Analytical Biochemistry*, 1976, **72**, 248.
6. Chan, K. -Y. and Wasserman, B.P., *Cereal Chemistry*, 1993, **70,** 22.
7. Clarkson, S.P. and Large, P.J., *Journal of the Institute of Brewing*, 1992, **98**, 111.
8. Doderer, A., Kokkelink, I., van der Veen, S., Valk, B.E. and Douma, A.C., Proceedings of the European Brewery Convention Congress, Lisbon, IRL:Oxford, 1991, p.109.
9. Ellman, G.L., *Archives of Biochemistry and Biophysics*, 1958, **74**, 443.
10. Ellman, G.L., *Archives of Biochemistry and Biophysics*, 1959, **82**, 70.
11. Graveland, A., Bosveld, P., Lichtendonk, W.J., Moonen, H.H.E. and Scheepstra, A., *Journal of the Science of Food and Agriculture*, 1982, **33**, 1117.
12. Hough, J.S., Briggs, D.D., Stevens, R. And Young, T.W., Malting and Brewing Science, 2nd ed., Chapman and Hall: London, 1981, Vol. 1, p.279
13. Jones, B.L. and Budde, A.D., Proceedings of the European

Brewery Convention Congress, Cannes, IRL: Oxford, 1999, p. 611.
14. Kaukovirta-Norja, A., Pöyri, S., Reinikainen, P., Olkku, J. and Laakso, S. Proceedings of the 25th Convention of the Institute of Brewing (Asia Pacific Section), Perth, Finsbury Press: Adelaide, 1998, p.196.
15. Koehler, S.M and Ho, T-H.D., *Plant Physiology*, 1990, **94**, 251.
16. Martel, C., Kohl, S. and Boivin, P., Proceedings of the European Brewery Convention Congress, Lisbon, IRL:Oxford, 1991, p. 425.
17. Matheis, G. and Whitaker, J.R., *Journal of Food Biochemistry*, 1987, **11**, 309.
18. Mikola, L. and Mikola, J., *Planta*, 1980, **149**, 149.
19. Moonen, J.H.E., Graveland, A. and Muts, G.C.J., *Journal of the Institute of Brewing*, 1987, **93**, 125.
20. Muts, G.C.J. and Pesman, L., European Brewery Convention Monographs XI, Wort Production, Maffliers, Verlag Hans Carl (Brauwelt-Verlag) :Nürnberg, 1986, p.25.
21. Muts, G.C.J., Kakeebe, M.G., Pesman, L. and van den Berg, R., Proceedings of the 18th Convention of the Institute of Brewing (Australia and New Zealand Section), Adelaide, Peacock Publications Kent Town, 1984, p. 115.
22. Phillips, H. and Wallace, W., *Phytochemistry*, 1989, **12**, 3285.
23. Poulle, M. and Jones, B.L., *Plant Physiology*, 1988, **88**, 1454.
24. Pöyri, S., Pettinen, P. and Home, S., Proceedings of the 26th Convention of the Institute of Brewing (Asia Pacific Section), Singapore, Hyde Park Press :Adelaide, 2000, p. 185.
25. Sjöholm, K., Pietilä, K., Home, S., Aarts, R. and Jaakkola, N., *Monatsschrift Brauwissenschaft*, 1994, **47**, 165.
26. Smith, D.B. and Lister, P.R., *Journal of Cereal Science,* 1983, **1**, 229.
27. Smith, D.B. and Simpson, P.A., *Journal of Cereal Science,* 1983, **1**, 185.
28. Thannhauser, T., Konishi, Y. and Scherega, H., Sulfur and Sulfur Amino Acids, In: Methods in Enzymology, W.B. Jakoby and O. W. Griffith Eds., 1987,Vol. 143, p. 115.
29. van den Berg, R. and van Eerde, P., Proceedings of the 17th Convention of the Institute of Brewing (Australia and New Zealand Section), Perth, 1982, p. 70.
30. van den Berg, R., Muts, G.C.J., Drost, B.W. and Graveland, A., Proceedings of the European Brewery Convention Congress, Copenhagen, IRL Press: Oxford, 1981, p. 461.
31. van Waesberghe, J., Int. Congr. Innovation in the Barley-Malt-Beer Chain, Nancy, France, Oct.16-17, 2000, p. 113.
32. Weiss, W., Postel, E. and Görg, A., *Electrophoresis,* 1992, **13**, 787.
33. Zhang, N. and Jones, B.L., *Journal of Cereal Science*, 1995, **21**, 145.

(Manuscript accepted for publication May 2002)