JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effective Prevention of Chill-Haze in Beer Using an Acid Proline-Specific Endoprotease from *Aspergillus niger*

MICHEL LOPEZ AND LUPPO EDENS*

DSM Food Specialties, Post Office Box 1, 2600 MA Delft, The Netherlands

Chill-haze formation during beer production is known to involve polyphenols that interact with proline-rich proteins. We hypothesized that incubating beer wort with a proline-specific protease would extensively hydrolyze these proline-rich proteins, yielding a peptide fraction that is unable to form a haze. Predigestion of the proline-rich wheat gliadin with different proteases pointed toward a strong haze-suppressing effect by a proline-specific enzyme. This finding was confirmed in small-scale brewing experiments using a recently identified proline-specific protease with an acidic pH optimum. Subsequent pilot plant trials demonstrated that, upon its addition during the fermentation phase of beer brewing, even low levels of this acidic enzyme effectively prevented chill-haze formation in bottled beer. Results of beer foam stability measurements indicated that the enzyme treatment leaves the beer foam almost unaffected. In combination with the enzyme's cost-effectiveness and regulatory status, these preliminary test results seem to favor further industrial development of this enzymatic beer stabilization method.

KEYWORDS: Beer colloidal stabilization; haze; polyphenol; haze active proteins; proline-specific protease

INTRODUCTION

During the production of beer and wines, both proteins and polyphenols are extracted from the disrupted plant tissue. Depending on their nature, ratios, and concentrations, these proteins and polyphenols may interact to form a haze. Whereas in wine, precipitate formation is mainly driven by the haze-active polyphenols, in beer, the haze-active protein fraction plays an important role (*1*).

The protein−polyphenol aggregate that can develop in bottled beer is referred to as chill-haze. Many studies have addressed chill-haze formation at molecular scale. It appears that a hydrophobic hordein fraction of malt combines with the polyphenols present to form a colloidal precipitate (*2*, *3*). Results from X-ray crystallography and NMR spectroscopy suggest that the protein−polyphenol interaction is driven initially by hydrophobic effects and is further stabilized by hydrogen bonding (*4*, *5*). Other publications illustrate the involvement of proline residues on the protein−polyphenol interaction phenomenon. For example, the haze-forming potential of proteins has been reported to be directly related to their proline content (*2*, *6*).

Several methods have been developed to prevent chill-haze formation in bottled beers. During beer maturation, a major part of the protein−polyphenol complexes is precipitated out by cooling the liquid. In the subsequent clarification process, either the remaining polyphenols or the remaining proteins are removed from the beer. Although PVPP is commonly used to remove remaining polyphenols, its use as a treatment does have a number of disadvantages, including the high capital costs for PVPP regeneration and the inherent lowering of the natural antioxidant potential of the beer. Removal of the remaining haze-active proteins is commonly achieved using silica gel. Although silica has been shown to specifically adsorb haze-active proteins (*7*), its protein absorbing capacity is limited so that this stabilizing method tends to be used for beers having moderate malt contents and requiring limited shelf stabilities (*8*).

An alternative option to prevent chill-haze formation in beer is the use of an acidic proteolytic enzyme such as papain. In this approach, broad spectrum proteolytic activity reduces the size of the remaining haze active proteins, probably yielding smaller protein−polyphenol aggregates with enhanced water solubility. Although the use of papain is relatively cheap, its proteolytic activity can impair beer foam (*9*).

Peptide bonds involving proline residues are notoriously difficult to cleave. This is illustrated by the fact that the currently available industrial proteolytic enzymes, including papain, cannot efficiently hydrolyze proline-rich proteins. Prolyl oligopeptidases (EC 3.4.21.26) represent a relatively recent addition to the group of serine proteases (*10*). These enzymes exhibit a narrow substrate specificity and have the unique possibility of preferentially cleaving peptides at the carboxyl side of proline residues. In a very recent paper, an exceptional debittering effect was described upon incubating casein hydrolyzates with a proline-specific protease (*11*). By reverse phase analysis, the debittering effect of the proline-specific protease on this mixture of proline-rich peptides could be linked to a significant reduction of the number of hydrophobic peptides present. Triggered by this observation, it was hypothesized that the same enzyme

* Author to whom correspondence should be addressed [telephone +31 15 279 3525; fax +31 15 279 3814; e-mail luppo.edens@dsm.com].

10.1021/jf0506535 CCC: $30.25
Published on Web 09/09/2005

would also effectively hydrolyze haze-active proteins, hereby drastically reducing the molecular weight and the hydrophobicity of the hordein fraction implicated in beer haze formation. As a result, chill-haze would be minimized. Because foam-active proteins are known to have only low proline contents (*12*), such an enzyme treatment would hardly affect beer foam stability.

To test this hypothesis, some initial model experiments were carried out using a readily available prolyl oligopeptidase with a neutral pH optimum (*13*). The results obtained in these studies encouraged us to invest in upscaling the production of the newly described prolyl endoprotease from *Aspergillus niger* with an acid pH optimum (*11*). Using the latter enzyme, several beer-brewing experiments were performed on pilot plant scale.

MATERIALS AND METHODS

Materials. Prolyl oligopeptidase from *Flavobacterium menigosepticum* was isolated as described by Diefenthal et al (*14*). The proline-specific endoprotease overproduced by *A. niger* was obtained as described (*11*). Recombinant neutral protease ("PNR") from *B. amyloliquefaciens* with a specific activity of 76 300 PC/g was provided by DSM Food Specialties (Delft, The Netherlands). Wheat gliadin and plant catechin were purchased from Sigma. The AEPA-1 turbidity standard was purchased from Haffmans (Venlo, The Netherlands). The PVPP used in the various experiments was Polyclar AT (ISP, NJ).

Enzyme Activity Tests. Activity measurements of the prolyl oligopeptidase from *F. meningosepticum* were performed on Z-Gly-Pro-pNA 0.26 mM in phosphate buffer 0.1 M pH 7.0 as described by Diefenthal et al (*14*). The enzymatic activity of the enzyme solution obtained was 40 unit/mL. Activity measurements of the *A. niger* proline-specific endoprotease were also performed on Z-Gly-Pro-pNA at 37 °C but in a 2 mM citric acid/disodium phosphate buffer pH 5.0. One enzyme unit was defined as the activity that liberates 1 μmol of pNA from Z-Gly-Pro-pNA in 1 min under the reaction conditions as described.

Gliadin−Catechin Model Experiments. Gliadin or gliadin-derived protein hydrolysates were dissolved at a concentration of 200 mL/L in a 0.02 M phosphate buffer at pH 7.0. The catechin solution was prepared by dissolution in 70% ethanol followed by dilution in 0.02 M phosphate pH 7.0 to reach 5.0 g/L in a solution containing 2% ethanol. The catechin solution was added at a rate of 2 mL/h in 4 mL of a stirred gliadin solution maintained at 25 °C. Mixing the catechin solution directly into 4 mL of phosphate buffer with 2% ethanol but without gliadin yielded the reference. Haze formation was measured during 20 min in a Tannometer (Pfeuffer GmbH, Germany) calibrated using a formazin standard solution prepared as described in Analytica-EBC method 9.29. The effects of the various proteases on gliadin−catechin hazes were established by preincubating the gliadin solution with one of the proteases used for 24 h at 45 °C.

Pilot-Scale Beer Production. At the 20 hL semi-industrial pilot brewery at the Institut Francais des Boissons de la Brasserie Malterie (IFBM, Vandoeuvre-les-Nancy, France), four all-malt beers were brewed under exactly the same conditions. Each brew was produced from 300 kg of barley malt. Mashing conditions of liquid/grist of 3:1 (vol/wt) and pH 5.6 were used. The mashing program has four phases: 45 °C for 20 min, 64 °C for 15 min, 74 °C for 30 min, and finally heating to 78 °C for 5 min. Between the phases, the heating rate was 1 °C per min. The wort was boiled 90 min with hop pellets added. Good trub separations were performed on whirlpool. Fermentation was carried out with the bottom-fermenting yeast strain Rh, as purchased from VLB (Berlin, Germany), using 17×10^6 viable cells/mL of wort. The fermentation process was at 12 °C until 5 Plato and at 14 °C until the end of fermentation. A cold maturation was carried out for 5 days at 1 ± 1 °C. The beers were carbonated to 5.2 g/L and pasteurized at 60 °C for 20 min.

The *A. niger*-derived prolyl endoprotease was added prior to the fermentation phases to reach final levels of either 25 units/hL (1.25 units/kg malt) or 50 units/hL (2.5 units/kg malt). PVPP stabilization was carried out at 30 g/hL and was mixed with kieselguhr during filtration.

Beer Haze Tests. Two different predictive shelf-life tests were used to predict the colloidal stability of the beers produced: the alcohol/low-temperature test according to Chapon (*15*) and the method recommended by the European Brewery Convention (Analytica-EBC section 9, method 9.30).

In the alcohol/low-temperature test, the haze stability of a beer is indicated by the density of the protein−polyphenol haze appearing upon incubating beer for 30 min at −8 °C after addition of 6% pure ethanol. According to the EBC predictive shelf-life test, bottled beer is stored overnight at 0 °C and for 48 h at 60 °C. Final turbidity is measured after another night at 0 °C. Each trial requires six measurements, that is, three bottles for the initial haze and another three bottles for the final haze measurements.

In the extended storage trials, beer turbidities were measured after storage of the bottled beers for periods up to 6 months at room temperature. The colloidal stabilities of these beers were measured using a Tannometer calibrated with AEPA-1 turbidity standards at 0.5 and 5.0 EBC.

Evaluation of Beer Foam Stability. Foam stability was assessed using the Ross and Clark method (*16*, *17*). This method provides a "Foam Stability Value", which is related to a foam quality scale: below 110, the foam is "bad"; between 110 and 119, the foam is "weak"; between 120 and 129, the foam is "satisfying"; between 130 and 139, the foam is "good"; and for any value above 140, the foam is considered to be "very good". All measurements are performed in triplicate.

Total Polyphenols in Beer. Total polyphenols in beer were measured by spectrophotometry as described in Analytica-EBC, section 9, method 9.11.

DPPH• − Reducing Power. The radical, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH•), shows color changes upon its reduction. An intensively colored free radical DPPH• was used to evaluate the reducing activity of beers produced according to Kaneda et al. (*18*) and Brand-Williams et al. (*19*). Decolorization of the DPPH• radical was measured at 525 nm at room temperature over time in the presence of reducing substances in an ethanolic/acetate buffer pH 4.3 2:1 (v/v) solution. The decreased absorbance after 10 min is directly proportional to antioxidant concentration.

RESULTS

Gliadin−Catechin Model Experiments. The gliadin−catechin interaction model has been widely used to study complex formation between proteins and polyphenols (*20*). By combining the plant polyphenol catechin with a wheat-derived, proline-rich, gliadin fraction, various parameters affecting the haze forming phenomenon can be investigated. This model was used to evaluate whether wheat gliadin prehydrolysis by different proteases leads to significant changes in the amount of gliadin−catechin haze formed. The gliadin fraction was therefore incubated with either a proline-specific or a "conventional" protease. The proline-specific protease used was the prolyl oligopeptidase as obtained from *Flavobacterium meningosepticum* (*14*). Because of the neutral pH optimum of the latter enzyme, we were obliged to study gliadin−catechin interactions under neutral pH conditions. The "conventional" enzyme used was a metallo-protease (EC 3.4.24.28) from *Bacillus amyloliquefaciens*, also optimally active under neutral pH conditions. Whereas the prolyl oligopeptidase is characterized by its strong preference for cleaving oligopeptides at the carboxy-terminal side of proline residues, the selectivity of the *Bacillus* enzyme is less stringent but cleavage of peptide bonds involving leucine, phenylalanine, or tyrosine residues is preferred.

After enzyme digestion, the two resulting wheat gliadin hydrolyzates were combined with the catechin solution according to the conditions of the gliadin−catechin interaction model. A nonhydrolyzed gliadin preparation and an incubation without gliadin were used as references. As expected, inubating catechin

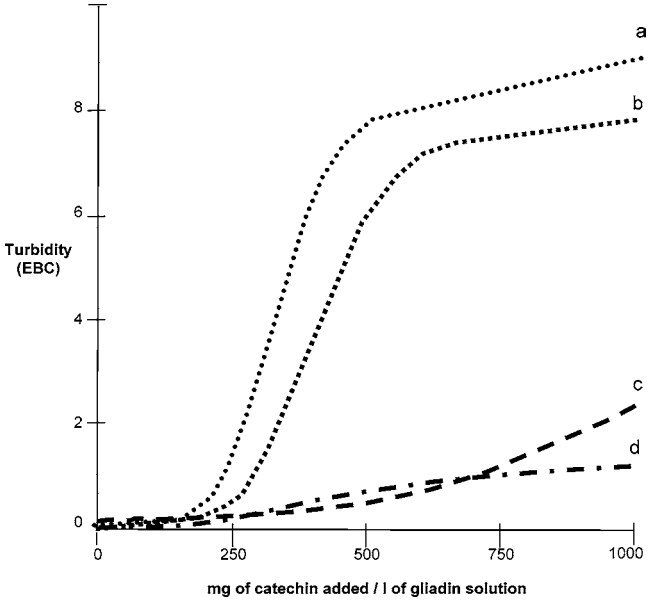

Figure 1. Haze formation profiles obtained by combining different gliadin preparations with various levels of catechin. Wheat gliadin was used as such (**a**) or after a preincubation with either the metalloprotease from *B. amyloliquefaciens* (**b**) or the proline-specific oligopeptidase from *F. meningosepticum* (**c**). The haze formed by adding just the buffer used for dissolving catechin to nonhydrolyzed gliadin is shown in (**d**).

with nonhydrolyzed wheat gliadin led to significant haze formation, whereas the incubation without gliadin did not. The combination of catechin and gliadin predigested with the metallo-protease also led to significant turbidity. However, predigestion of gliadin with the prolyl oligopeptidase completely prevented haze formation (**Figure 1**). This result highlighted indications that a proline-specific protease can markedly affect gliadin−catechin interaction. Furthermore, this observation lends additional support to the notion that proline residues play an important role in the aggregate forming phenomenon.

Performance of the *A. niger*-Derived Enzyme in Beer. The overexpression of a proline-specific protease from the food grade microorganism *A. niger* was described recently (*11*). In a manner similar to known prolyl oligopeptidases, this enzyme has a strong preference for cleaving at the C-terminal side of proline residues but exhibits optimal activity around pH 4. Other differences with the known prolyl oligopeptidases are that the *A. niger* enzyme is secreted into the fermentation broth, hereby facilitating its recovery in an almost pure state. Additionally, the *A. niger*-derived enzyme is a true endoprotease. Whereas the known proline-specific oligopeptidases are so-called oligopeptidases unable to cleave peptides of more than 30 amino acids long, the proline-specific enzyme from *A. niger* can hydrolyze peptides as well as intact proteins. These unusual properties in combination with a very limited sequence homology with the known prolyl oligopeptidases suggest that the *A. niger*-derived enzyme represents a new type of proline-specific endoprotease.

The effectiveness of this *A. niger*-derived, acid proline-specific endoprotease was tested in a number of small-scale beer-brewing experiments, once larger quantities were available. The outcome of these experiments reconfirmed earlier observations regarding the remarkable effectiveness of a proline-specific protease toward haze prevention. Therefore, we decided to initiate a number of pilot-scale beer-brewing experiments.

Colloidal and Foam Stability of Enzyme-Treated Beers Produced on 20 hL Pilot Scale. In three 20 hL beer productions, two different dosages of the *A. niger*-derived prolyl endoprotease were tested. In two of these productions, the same enzyme dosage was applied to test the reproducibility of the method. In all cases, the enzyme was added just before the start of the beer fermentation. No other stabilizing agents were used in combination with the enzyme. The reference beer was stabilized with PVPP (30 g/hL) in a conventional way. Because it is likely that the quantity of malt used largely determines the level of haze active proteins present, the enzyme dosages used were calculated per kg of malt added. The lowest enzyme dosage used was 1.25 units/kg malt (25 units/hL), and the highest dosage used was 2.5 units/kg malt (50 units/hL). Immediately after bottling, the various beers were subjected to two different forcing tests: the EBC predictive shelf-life test and the alcohol-chill test as developed by Chapon (*15*).

The data presented in **Table 1** show that both tests predict excellent shelf stabilities for beers produced with the proline-specific endoprotease. Even at the lowest enzyme activity level tested, both the EBC forcing test and the alcohol-chill tests predict a stabilization effect that is superior to a conventional PVPP treatment. The independent production runs using identical enzyme dosages (2.50 unit/kg) also yielded comparable haze data.

Subsequent stability tests of the bottled beers stored at room temperature for periods up to 6 months further substantiated our findings. The graphs shown in **Figure 2** illustrate that the enzyme-treated beers are almost completely stable at room temperature for storage periods up to 6 months. When using an enzyme dosage of 2.5 units/kg malt, beer turbidity remains below 1 EBC during this whole period. A dosage of 1.25 units/kg malt results in a beer turbidity around 1.5 EBC but without a significant turbidity increase during the six months storage period. Using a stabilized and concentrated enzyme solution, the latter enzyme dosage (corresponding with 25 units/hL of an all-malt wort) would imply adding just a few liters of enzyme concentrate per 1000 hL of beer. In contrast with the enzyme treatment, the turbidity of the PVPP-treated beer steadily increased during the first 4 months of storage to reach a value of 2 EBC units.

Although beer foam proteins are known to contain only low levels of proline residues (*12*), lengthy incubations with the proline-specific endoprotease could negatively influence beer foam stability. To quantify such possible negative effects, the foam stabilities of the beers produced according to the above-described protocols were measured using the Ross and Clark method (*17*), which states that the quantity of liquid that can be drained from a beer foam characterizes beer foam stability. "Very good" foams score "Foam Stability Values" above 140, "good" foams score between 130 and 139, and "satisfying" foams score between 120 and 129. Foam stabilities were measured immediately after bottling and also after 4 and 6 months storage at room temperature. According to the data presented in **Figure 3**, the foam of the PVPP stabilized beer is rated "very good" after 4 months and "good" after 6 months of storage; a similar rating is obtained for the beer treated with 1.25 enzyme units per kg malt. Beers treated with 2.50 enzyme units per kg malt reach a "satisfying" score after 6 months of storage. These data indicate a slightly greater decrease in foam in the enzyme-treated than in the PVPP-treated beer.

Effects on Polyphenol Levels. Because PVPP treatment selectively removes residual polyphenols and the proline-specific protease prefers to hydrolyze haze-active proteins, it may be expected that beers stabilized by the proline-specific protease will have higher polyphenol levels than beers stabilized by a

Table 1. Predictive Shelf-Life Tests Carried out on 100% Malt Beers Produced at 20 hL Scale and Stabilized with Either PVPP or by Proline-Specific Endoprotease and Measured Immediately after Bottling

		EBC forcing test		alcohol-chill test
colloidal stabilization of the beers		initial haze (EBC): turbidity after one night at 0 °C	final haze (EBC): turbidity after one night at 0 °C, 48 h at 60 °C, and one night at 0 °C	haze (EBC): turbidity with 6% ethanol and kept 40 min at –8 °C
PVPP	30 g/hL of beer	3.0	8.9	79
proline-specific endoprotease	1.25 unit/kg	1.9	8.9	48
	2.50 unit/kg	1.4	6.0	45
	2.50 unit/kg	1.5	7.0	36

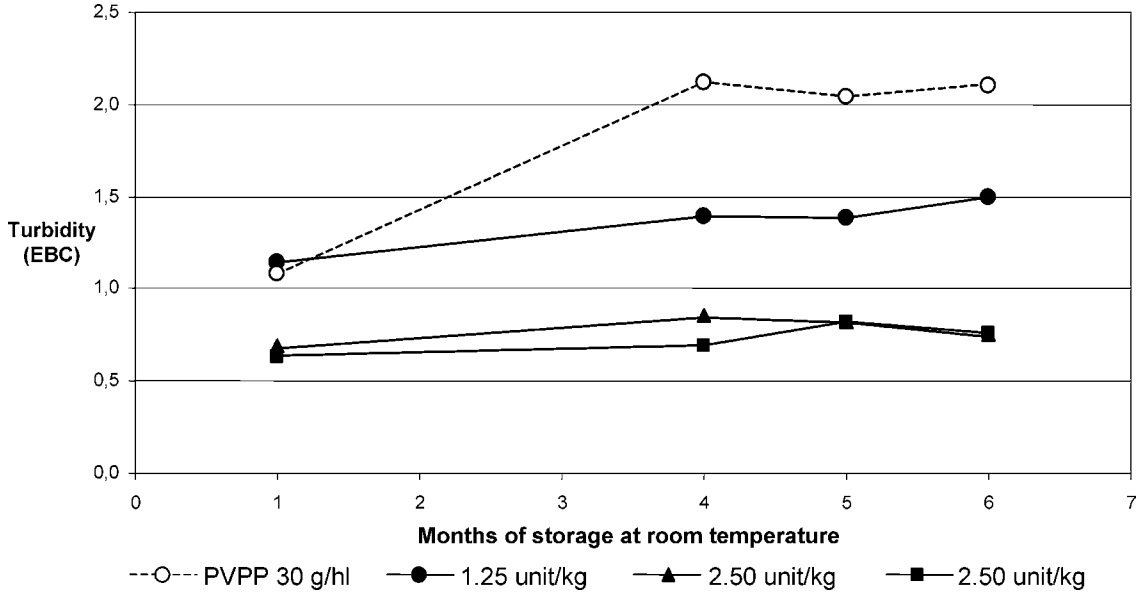

Figure 2. Colloidal stability of bottled beers stored at room temperature for 1, 4, 5, and 6 months. The beers were produced at 20 hL scale and stabilized with either PVPP or the proline-specific endoprotease. Turbidities were measured using a tannometer calibrated with different AEPA-1 turbidity standards.

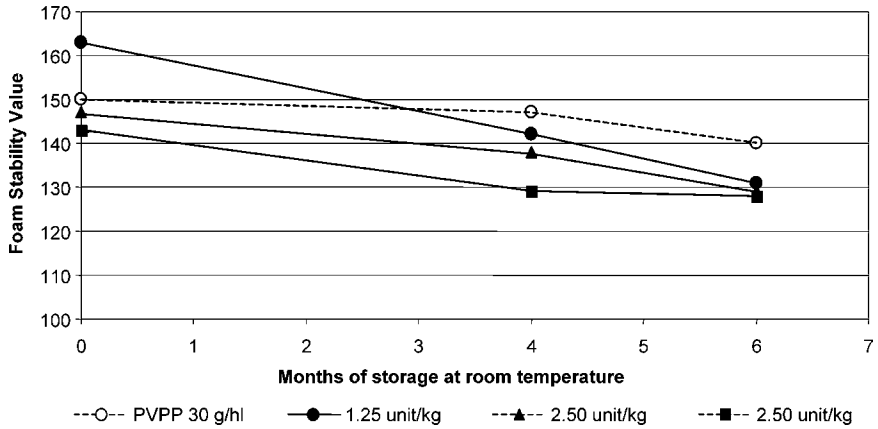

Figure 3. Foam stability of beers upon bottling or stored at room temperature for 4 and 6 months. The beers were produced at 20 hL scale and stabilized with either PVPP or the proline-specific endoprotease.

PVPP treatment. As shown in **Figure 4**, PVPP-treated beer contains approximately 125 mg/L polyphenol in comparison to enzyme-treated beers that contain approximately 200 mg/L. These figures seem to agree with earlier observations showing that PVPP treatment removes approximately one-half of the haze-active polyphenols (*7*). Such reductions in polyphenol levels have been linked with diminished reducing capacities of beers (*21*). To quantify the difference in reducing capacities between the enzyme-treated and PVPP-treated beers, a test with 1,1-diphenyl-2-picrylhydrazyl (DPPH•) was carried out (*18, 19*). As shown in **Figure 4**, the enzyme-treated beers reduce the DPPH• radical more rapidly than the PVPP stabilized beer, reconfirming the higher polyphenol levels in the enzyme stabilized beers. However, these results cannot be related to improved flavor stabilities (*21*).

DISCUSSION

In this paper, the benefits of a proline-specific protease in beer stabilization have been demonstrated for the first time. The required enzymatic activity was provided by a recently described acid proline-specific endoprotease obtained from the food grade

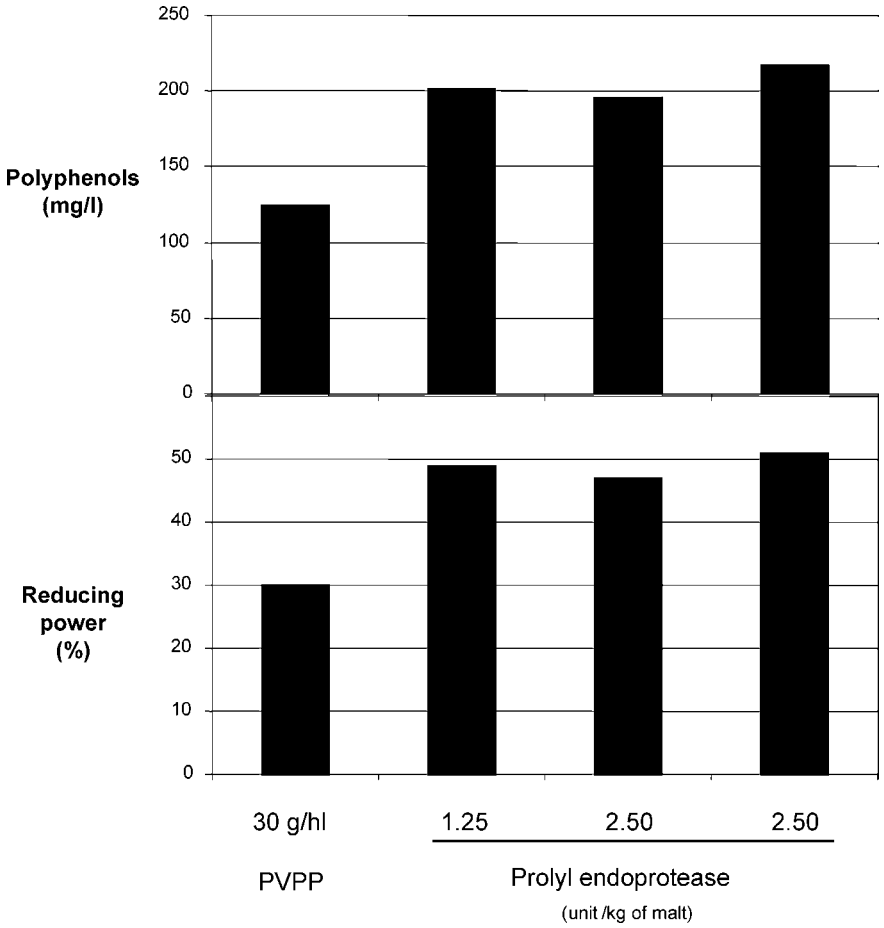

Figure 4. Polyphenol levels of beers produced at 20 hL scale and stabilized with either PVPP or the proline-specific endoprotease. The antioxidant potential of the various beers was assessed by quantifying their reducing power upon a challenge with the free radical DPPH•.

microorganism *A. niger*. The narrow substrate specificity of this enzyme targets its hydrolytic activity toward proline-rich, that is, haze active proteins. Foam stabilizing proteins featuring low proline contents form poor substrates for the enzyme.

The starting point was an analogy with the debittering effects observed with protein hydrolysates (*11*), indicating that the prevention of beer haze could be connected with an extensive hydrolysis of proline-rich proteins. By drastically reducing their chain lengths, haze-active proteins will become less hydrophobic and cannot contribute to forming large protein−polyphenol networks.

Our data show that surprisingly low dosages of the proline-specific protease could stabilize beer as effectively as the conventionally used PVPP, but yielding a final product containing higher polyphenol levels and featuring higher reducing capacities than PVPP-treated beers. Adverse effects of the enzyme on beer foam stability were almost absent. Apart from providing adequate stabilization, the enzymatic approach could also offer significant processing advantages. For example, the stabilization of 1000 hL of an all-malt beer would require the dosing of not more than a few liters of enzyme concentrate. Whereas PVPP and silica treatments are carried out in a process area very sensitive to oxygen ingress, the enzyme solution cannot only be added during the fermentation phase of the process, but obviates the needs for the disposal of spent materials.

Clearly, more tests need to be carried out to validate our hypothesis that the enzyme treatment represents a realistic and economical alternative to the existing beer stabilization methods. For example, some residual enzymatic activity was noted in the bottled beer produced in the IFBM pilot facilities. Furthermore, real production scale stability data are still lacking as well as an adequate insight into the organoleptic effects on different beers. However, enzyme-wise there seem to exist few restrictions for a further industrial development. The *A. niger* host used for overexpressing the *A. niger* gene encoding the relevant protease is a food grade microorganism and a recognized producer of a number of enzymes enjoying GRAS status. Ongoing enzyme production trials also show that during large-scale fermentation the enzyme is secreted in considerable quantities in an almost pure form. Therefore, we are confident that from legislative and economic points of view this new beer stabilization approach will become within reach of the beer industry.

ACKNOWLEDGMENT

We thank Sylvain Delaroche and Jean-Marc Derquenne, DSM Food Specialties, for their excellent technical support. We especially thank Patrick Boivin and Mbaka Malanda, Institut Français des Boissons de la Brasserie et de la Malterie − IFBM, Vandoeuvre-Les-Nancy, for their pilot-scale experiments and helpful discussions on the various beer analyses.

LITERATURE CITED

(1) Siebert, K. J.; Carrasco, A.; Lynn, P. Y. Formation of protein-polyphenol haze in beverages. *J. Agric. Food Chem.* **1996**, *44*, 1997−2005.

(2) Asano, K.; Shinagawa, K.; Hashimoto, N. Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J. Am. Soc. Brew. Chem.* **1982**, *40*, 147−154.

(3) O'Rourke, T. Brewing. In *Industrial Enzymology*, 2nd ed.; Godfrey, T., West, S., Eds.; MacMillan Press Ltd.: London, UK, 1996; pp 124−125.
(4) Oh, H. I.; Hoff, J. E.; Armstrong, G. S.; Haff, L. A. Hydrophobic interaction in tannin-protein complexes. *J. Agric. Food Chem.* **1980**, *28*, 394−398.
(5) Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205−215.
(6) Siebert, K. J. Effects of protein-polyphenol interactions on beverage haze, stabilization, and analysis. *J. Agric. Food Chem.* **1999**, *47*, 353−362.
(7) Siebert, K. J.; Lynn, P. Y. Mechanisms of beer colloidal stabilization. *J. Am. Soc. Brew. Chem.* **1997**, *55*, 73−78.
(8) Bamforth, C. W. Beer haze. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 81−90.
(9) Posada, J.; Almenar, J.; Garcia Galindo, J. A practical approach on protein stabilizers. *Proc. - Eur. Brew. Conv.* **1971**, *13*, 379−391.
(10) Barrett, A. J.; Rawlings, N. D. Oligopeptidases and the emergence of the prolyl oligopeptidase family. *Biol. Chem. Hoppe-Seyler* **1992**, *373*, 353−360.
(11) Edens, L.; Dekker, P.; van der Hoeven, R.; Deen, F.; de Roos, A.; Floris, R. An extracellular prolyl endoprotease from *Aspergillus niger* and its use in the debittering of protein hydrolysates. *J. Agric. Food Chem.* **2005**, *53*, 7950−7957.
(12) Leiper, K. A.; Stewart, G. G.; McKeown, I. P. Beer polypeptides and silica gel, Part II. Polypeptides involved in foam formation. *J. Inst. Brew.* **2003**, *109*, 73−79.
(13) Diefenthal, T.; Dargatz, H.; Witte, V.; Reipen, G.; Svendsen, I. Cloning of proline-specific endopeptidase gene from *Flavobacterium meningosepticum*: expression in *Escherichia coli* and purification of the heterologous protein. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 90−97.
(14) Diefenthal, T.; Dargatz, H. Rapid purification of proline-specific endopeptidase from *Flavobacterium* meningosepticum heterologously expressed in E. coli. *World J. Microbiol. Biotechnol.* **1995**, *11*, 209−212.
(15) Chapon, L. Nephelometry as a method for studying the relations between polyphenols and proteins. *J. Inst. Brew.* **1993**, *99*, 49−56.
(16) Ross, S.; Clark, G. L. *Wallerstein Lab. Commun.* **1939**, *2*, 46.
(17) De Clerk, J. Mesure de stabilité de mousse. *Cours de Brasserie*, 2nd ed. **1963**, *II*, 746−752.
(18) Kaneda, H.; Kobayashi, N.; Furusho, S.; Sahara, H.; Koshino, S. Reducing activity and flavor stability of beer. *MBAA Tech. Q.* **1995**, *32*, 90−94.
(19) Brand-Williams, W.; Cuvelier M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25−30.
(20) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol-protein interactions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.
(21) McMurrough, I.; Madigan, D.; Kelly, R. J. The role of flavanoid polyphenols in beer stability. *J. Am. Soc. Brew. Chem.* **1996**, *54*, 141−148.

Received for review March 23, 2005. Revised manuscript received July 6, 2005. Accepted July 14, 2005.

JF0506535