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On the Influence of the Malting and Brewing Process on the Amounts of Pronyl-L-Lysine, a Chemopreventive and Antioxidant Maillard Product in Melanoidins

The influence of the type of malt, the malting procedure, as well as the brewing process on the amounts of the antioxidant and Phase II-Enzyme modulating, protein-bound 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2*H*-pyrrol, known as pronyl-L-lysine, in malts and beers was investigated in quantitative studies. These studies revealed a strong influence of both the malting and brewing processes on the contents of the chemopreventive target compound in the final products. This knowledge might be helpful to systematically increase of the amounts of the bioactive, protein-bound pronyl-L-lysine in beer and beer containing beverages by technological means.

Descriptors: Pronyl-L-lysine, melanoidins, beer, malt, reducing power, colour, TBN

1 Introduction

During the manufacturing of malt from barley, a complex cascade of non-enzymatic reactions, the so-called Maillard reaction, takes place between reducing carbohydrates and proteins. The Maillard Reaction is chiefly responsible for the development of the aroma and the typical brown colouration of the malt as well as the final beer produced thereof. Although it is generally accepted that the coloured compounds formed during malt manufacturing belong to the group of Maillard-derived, brown coloured macromolecules, the so-called melanoidins, the knowledge about their exact chemical structures as well as the health effects of these components is relatively scarce.

Very recently, the chemopreventive action of thermally treated food products such as bread crust or kilned malt was reported in intestinal Caco-2 cells which showed an induction of the Phase II biotransformation enzyme glutathione-*S*-transferase (GST) after exposure to aqueous extracts prepared from bread crust [1] and malt [2], respectively. Since induction of GST or other Phase-II enzymes by, i.e. antioxidants represents a promising strategy for cancer prevention, activity-guided fractionation of food melanoidins as well as suitable Maillard-type model systems has been recently performed to identify the melanoidin structures which may function as a Phase-II inducer in biological systems. These studies led to the successful identification of the protein-bound 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2*H*-pyrrol (pronyl-L-lysine; I in Figure 1) as part of melanoidins present in bread crust [1], malt [3], and beer [3], and demonstrated for the first time that such "pronylated" proteins act as monofunctional inducers of GST, serving as a functional parameter of an antioxidant, chemopreventive activity *in vitro*. Model studies revealed that the pronyl-L-lysine is formed during thermal treatment by Maillard reactions from glucose liberated from starch, maltooligosaccharides, and maltose, respectively, and lysine side chains or the N-terminus of the proteins via the transient intermediate and penultimate precursor acetylformoin (Fig. 1).

Recently, animal feeding studies with malt extract and pronylated proteins,demonstrated that the GST activity in the liver of rats was increased when compared to the control group [3]. For example, GST activity was elevated by 27 % in the liver of animals administered pronylated proteins. In addition, the plasma tocopherol level was increased by about 14 % when malt or pronyl-BSA were fed, while the levels of malondialdehyde were decreased and the total antioxidant capacity was increased. In consequence, the main systemic effects of dietary pronylated proteins were, for the first time, demonstrated to be an enhanced antioxidant capacity and the particulate increase in chemopreventive enzymes [3].

Although protein-bound pronyl-L-lysine was identified in malt and beer [3], there is no data available on how the content of pronyl-L-lysine is influenced by the technological processes of malting and brewing. This information is, however, the necessary prerequisite to technologically increasing the amounts of the bioactive pronyl-L-lysine in malts and final beers.

The objectives of the present investigation were, therefore, to quantify the amounts of pronyl-L-lysine in malts prepared by different kilning procedures, and to quantitatively study the influence of the manufacturing process from barley to the ready-to-drink beer on pronyl-L-lysine content in the final products.

2 Material and Methods

2.1 Malt

The malting was performed in a 1 kg micro-scale. To produce comparable malt samples, a standard malting procedure was used as described in MEBAK, Vol. 1, Method 2.5.3.1. [4]. For variations in the germination time, the standard procedure mentioned

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Tables and Figures see Appendix

above was used but the kilning started after 0.5, 3, 5, and 7 days of germination, respectively. The kilning procedures with different kilning-off temperatures are shown in Figure 2.

Withering temperature of 50 °C was held for 16 h, thereafter the temperature was increased stepwise by 10 °C per hour until kilning-off temperature was reached. To record the influence of the duration of different kilning-off temperatures, samples were taken at three different time intervals for each temperature (70 °C: 5.25, 10.5, and 15.25 h; 80 °C: 2.5, 5, and 7.5 h; 90 °C: 1.25, 2.5, and 3.75 h). All other malts used were commercial products obtained from Malzfabrik Weyermann, Bamberg, Germany.

2.2 Mashing procedures

Congress mashing: Congress mashing was carried out closely following the method of MEBAK (Fig. 3), Vol. 1, Method 4.1.4.2. [4]

All following mashing procedures were carried out in congress scale on the basis of 50 g barley malt and 250 g distilled water.

Cold water extraction: Ground malt (0.2 mm i.d., according to MEBAK, Vol. 1, Method 4.1.4.2 [4]) and water were mixed, stirred for 30 min at 25 °C, and filtered (Macherey-Nagel MN 514 ¼, Düren, Germany).

Infusion mashing: As shown in Figure 3, two different procedures were used for infusion mashing. Mashing-in temperature of the first procedure (“infusion 45”) was 45 °C, and was held for 30 min. Thereafter, the temperature was increased to 62, kept for 30 min, then increased to 72 °C, and again maintained for 30 min. Finally, the temperature was increased to 76 °C and held for 10 min. In the second procedure (“infusion 62”), we started with a higher initial temperature of 62 °C maintained for 45 min, thereafter increasing the temperature to 72 °C, kept for 30 min, and, finally, increased to 76 °C for 10 min.

2.3 Brewing process

After milling with a 2-roll mill (0.8 mm roll distance), the mashing was performed using the “Infusion 62” procedure. Grist-water ratio was 1:4. The details of the parameters applied for beer preparation are given in Table 1.

2.4 Colour

In order to prepare three different beers exhibiting the same colour intensity, three different brews were prepared by mixing different types of malt. One beer was produced from 100 % Vienna malt which gave the base colour. The two additional beers were prepared from Pilsner malt/Munich malt and Pilsner malt/Caramel malt mixtures.

Colour measurements were recorded at 430 nm with a spectral photometer (CADAS 200, Dr. Lange, Berlin, Germany) according to the EBC-Method 8.5. [5] When necessary, appropriate dilutions of the samples were made.

2.5 Quantitative analysis of pronyl-L-lysine

Liquid samples: Water (150 ml) and methylhydrazine (1.2 ml) were added to the liquid sample (50 ml), the pH-value of the mixture was adjusted to 4.0 with conc. HCl, and then maintained for 1h at 80°C. After cooling to room temperature with an ice bath, the pH-value was adjusted to 7.0 with NaOH (2 mol/l), and the mixture was then extracted with dichloromethane (3 x 250 ml). The combined organic layers were centrifuged (10000 rpm), the supernatant was then concentrated in vacuum (25 °C, 850 mbar) to a final volume of 2 mL, and then submitted to quantitative analysis as reported recently [1].

Solid samples: Malt samples were frozen with liquid nitrogen, ground by means of an ultracentrifuge mill (Retsch, Haan, Dermany), aliquots of the malt powder (24 g) were suspended in water (250 ml), and methylhydrazine (3 mL) was added. After maintaining the mixture for 1 h at 80°C, the sample clean-up was done as described above.

2.6 Reducing power

The determination of the reducing power of beer samples was performed by means of a Tannometer (Pfeuffer, Kitzingen, Germany) based on the decolourisation of DPPH as described in MEBAK, Vol. 2, Method 2.20.1. [6]

2.7 TBN

The determination of the Thiobarbituric Acid Number (TBN) was done closely following the method described in MEBAK, Vol. 2, Method 2.4. [6]

2.8 Carbohydrates

Fermentable carbohydrates were quantified according to the method of Kessler [7] using high-performance anion exchange chromatography (Dionex Cooperation, Sunnyvale, USA) with pulsed amperometric detection (HPAEC-PAD).

3 Results and discussion

3.1 Influence of germination time on pronyl-L-lysine contents in malt

In order to get a first insight into the influence of germination time on the content of chemopreventive and antioxidant melanoidins in kilned-off green malt, the kilning of the green malt was done after 0.5, 3, 5, and 7 days of germination, and the amounts pronyl-L-lysine were quantitatively determined. As shown in Figure 4, the levels of the antioxidant increased from 0.01 mg/L for kilned barley to a maximum of 0.24 mg/L for the malt sample which was germinated for five days prior to kilning. Further increase of the germination time resulted again in an increase of pronyl-L-lysine levels.

As reducing carbohydrates are known to be the precursors of protein-bound pronyl-L-lysine in the reaction with barley proteins, fructose, glucose, sucrose, maltose and maltotriose were quantitatively determined in these malt samples. As given in Figure 4, the influence of the germination time on the total amount of these sugars collaborated well with the quantitative data found for pronyl-L-lysine, thus confirming these low-molecular weight sugars as the key progenitors of pronyl-L-lysine. In particular the levels of the reducing carbohydrates fructose, maltose, and maltotriose increased strongly up to a germination time of 5 days (Table 2).

The results obtained from these studies clearly indicate that the pronyl-L-lysine content is significantly influenced by germination time, reaching a maximum after five days of germination.

3.2 Influence of kilning-off temperature and time on pronyl-L-lysine contents in malt

To study the influence of the kilning-off temperature and the kilning-off time on the concentrations of pronyl-L-lysine in malt, samples were taken at 5.25, 10.5, and 15.25 h at 70, 80, and 90°C, respectively, and the amounts of the antioxidant were determined quantitatively. As given in Figure 5, the lowest amounts of pronyl-L-lysine were found after withering. With increasing the duration and the kilning-off temperature, we observed a favoured pronyl-L-lysine generation. It is interesting to note that the TBN values measured for these samples showed a significant correlation (R = 0.940***) with the concentration of pronyl-L-lysine (Figs. 5 and 6).

In addition, pronyl-L-lysine content seem to correlate with the colour of congress wort (data not shown) and the boiled congress wort (R = 0.915***), thus giving evidence that darker malts contain higher amount of pronyl-L-lysine than pale malts.

3.3 Pronyl-L-lysine contents in commercial malts and influence of mashing procedure

To confirm the data obtained above and to evaluate the impact of the mashing procedure on the pronyl-L-lysine content, mashes were produced starting from three different malt types and applying three different mashing procedures. Quantitative analysis of pronyl-L-lysine in these nine samples revealed that the dark coloured Caramel malt, followed by the medium dark coloured Munich malt contained the highest levels of pronyl-L-lysine, whereas the pale Pilsner malt just contained minor amounts of the antioxidant (Fig. 7).

Also the mashing procedure was found to influence pronyl-L-lysine contents. Compared to the "Infusion 45", a major amount of pronyl-L-lysine could already be solubilised by means of a cold water extraction (Fig. 7). However, no large difference was observable between the malts prepared with mashing-in temperatures of 45 and 62 °C. These results clearly demonstrate that there is no significant impact of enzymatic activity on the release of pronyl-L-lysine from melanoidins. Again, this data confirms that dark or higher kilned-off malts yield more pronyl-L-lysine than pale malts.

3.4 Influence of pH of the mashing process on pronyl-L-lysine contents

In order to study the influence of the pH value on pronyl-L-lysine content in mashes, Munich malt mash was adjusted to pH 4.02, 5.01, and 5.98 by adding lactic acid or sodium hydroxide at the beginning of the mashing process, thus reaching final pH-values of 4.11, 5.24 and 5.79, respectively. Quantitative analysis of pronyl-L-lysine revealed higher pronyl-L-lysine contents when mashing was done at the lowest pH value (Fig. 8). With increasing pH value of the mash, a slight increase in colour and no significant change in reducing power was observed.

The observed effect of the pH value on the concentrations of pronyl-L-lysine in mash has been confirmed by analysis of ten different malts (data not shown). As Maillard reactions are not expected to take place under the low-temperature conditions applied in these experiments in a large extent, the increase of pronyl-L-lysine contents with lowering the pH value cannot be explained by its *de novo* generation from carbohydrates and proteins, but by the recently observed increased chemical stablility of pronyl-L-lysine at lower pH values (data not published).

3.5 "Iso-Colour"

It has been shown that the type of malt is the most relevant factor determining the amounts of pronyl-L-lysine entering the brewing process to give the final beer. In order to investigate whether it is possible to produce beers exhibiting the same colour intensity but varying pronyl-L-lysine concentrations, we produced three iso-coloured beer samples (11-13 EBC units), one from 100% Vienna malt, and two sample from binary mixture of Pilsner malt, Munich malt, and Caramel malt, respectively (Table 3).

First, pronyl-L-lysine was quantitatively determined in samples obtained from different process steps from the three different malt mixtures (Fig. 9). Independent from the processing step, the Vienna malt contained by far the highest amounts of pronyl-L-lysine, whereas the lowest amounts are found in the binary Pilsner malt/Caramel malt mixture.

3.6 Brewing process

To get a better understanding of the behaviour of pronyl-L-lysine during the brewing process, quantitative analysis of the antioxidant was done in several intermediate steps from the mash of a pale and a dark malt, to the corresponding beers. As given in Figure 10, an increase of pronyl-L-lysine content was found during wort boiling. Thereafter up to the fresh beer there are only minor changes in pronyl-L-lysine concentrations. Even after three month of storage, no significant changes in pronyl-L-lysine content could be observed (Fig. 10).

It is interesting to note that starting from the Pilsner malt a drastic increase in pronyl-L-lysine content by more than 400 % was observed from the end of mashing to the fresh beer (Fig. 10). In comparison, starting with Munich malt just resulted in an increase in pronyl-L-lysine concentration by about 25 % only. This finding might be explained by the fact that Pilsner malt wort still contains major amounts of reducing carbohydrates, proteins, and amino acids which can be converted into pronyl-L-lysine during wort boiling, whereas in Munich malt major amounts of these precursors have already been thermally consumed during the kilning-off procedure. However, comparing the absolute amounts of pronyl-L-lysine in both beers clearly demonstrated that the beer produced from Munich malt contained more than twice of the amount of pronyl-L-lysine than the beer made from Pilsner malt (Fig. 10).

4 Conclusion

The data obtained by this study show first evidence that pronylated proteins (I in Fig. 1), recently reported to exhibit antioxidant and chemopreventive activity *in vivo*, as part of the coloured melanoidins in malts are influenced by the type of malt, the mashing procedure, as well as the brewing process. Based on this important information, future investigations might focus on the systematic increase of the amounts of the bioactive protein-bound pronyl-L-lysine in beer and beer-containing beverages by technological means.

5 Acknowledgement

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6 Literature

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Appendix

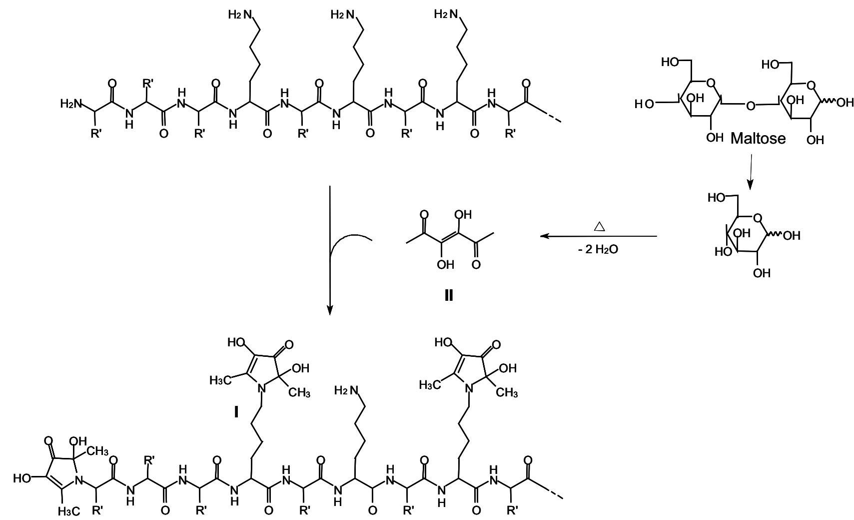

Fig. 1 Formation pathway leading to pronyl-L-lysine (I) during malt kilning from maltose and proteins via the key intermediate acetylformoin (II)

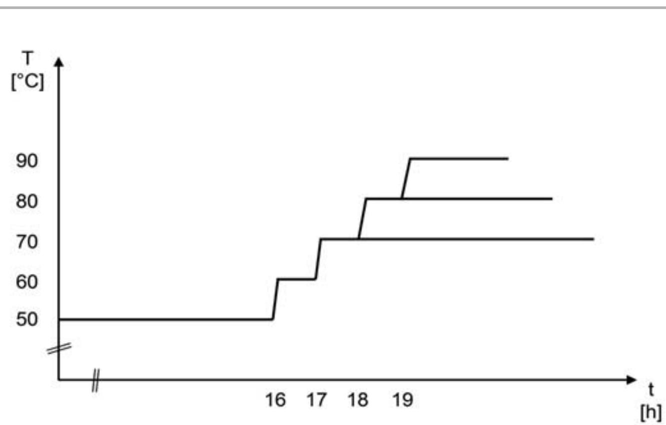

Fig. 2 Withering and kilning procedure with different kilning-off temperatures

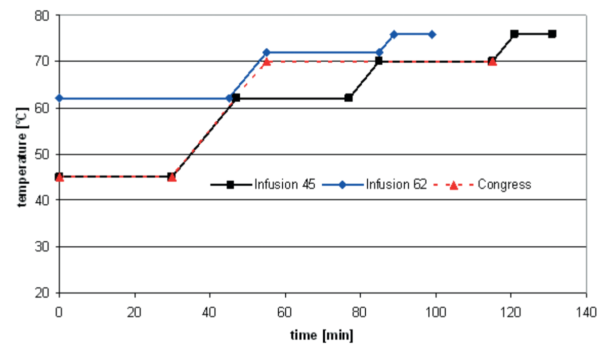

Fig. 3 Time-temperature profile of different mashing procedures

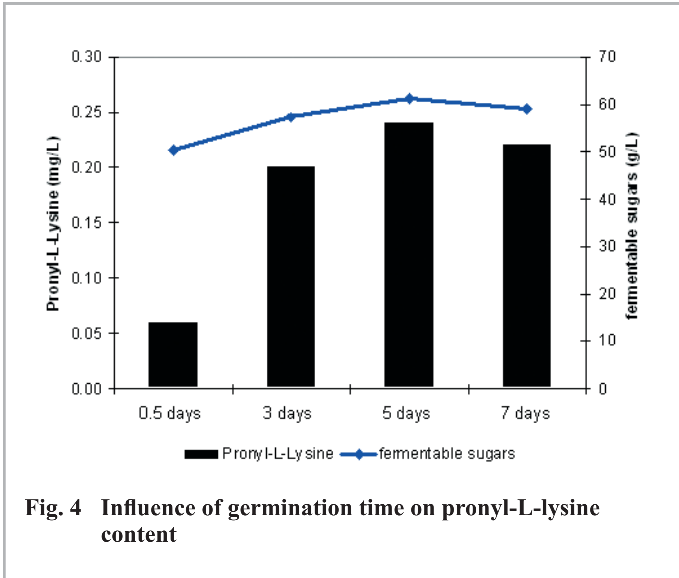

Fig. 4 Influence of germination time on pronyl-L-lysine content

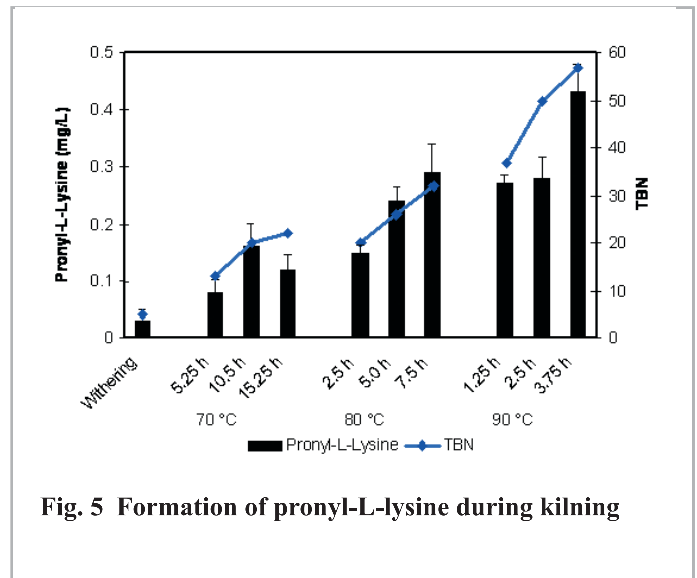

Fig. 5 Formation of pronyl-L-lysine during kilning

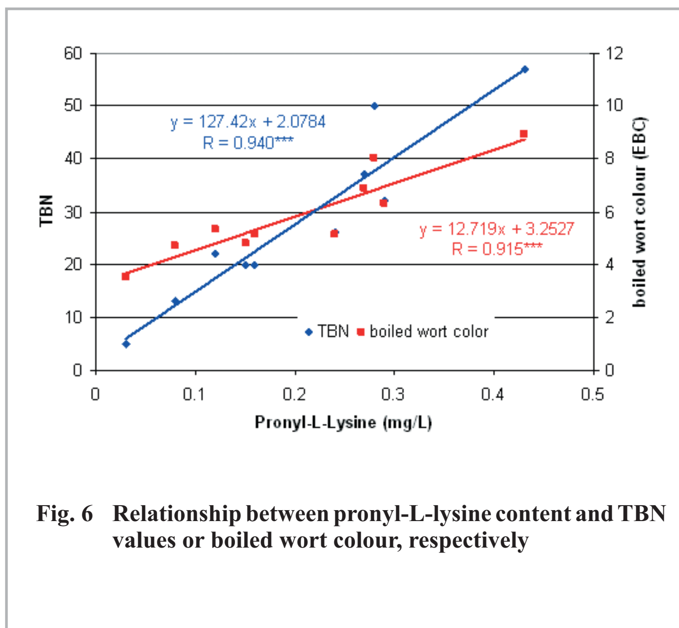

Fig. 6 Relationship between pronyl-L-lysine content and TBN values or boiled wort colour, respectively

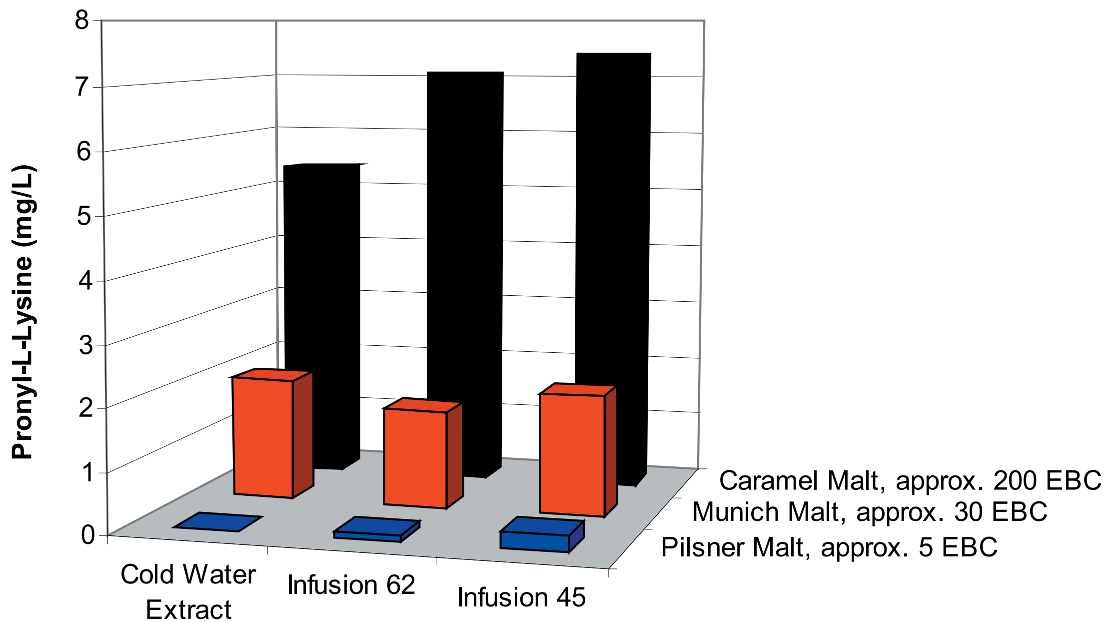

Fig. 7 Pronyl-L-lysine content of different malts

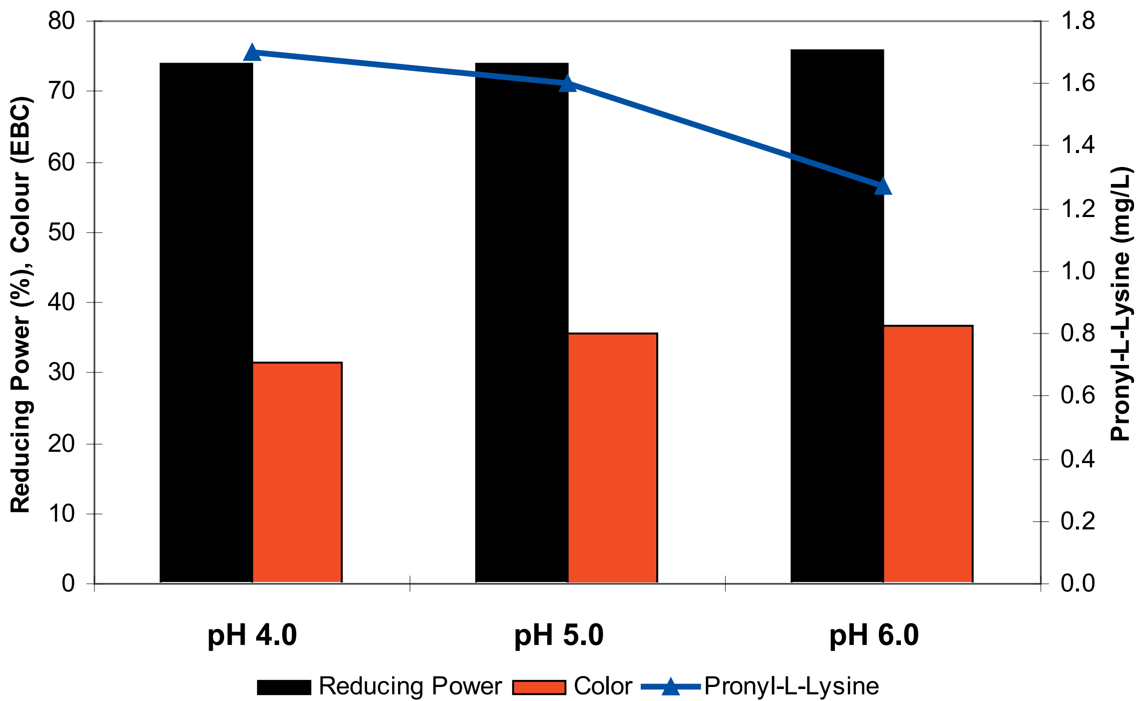

Fig. 8 Effect of pH value adjusted by lactic acid and NaOH

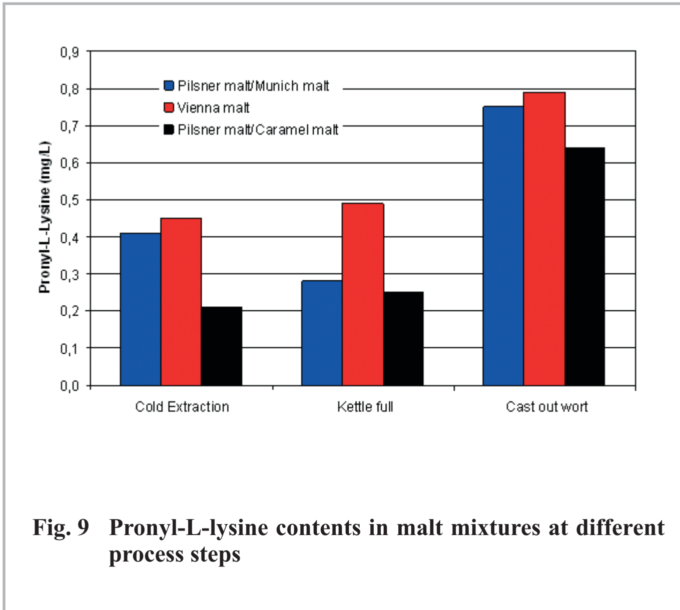

Fig. 9 Pronyl-L-lysine contents in malt mixtures at different process steps

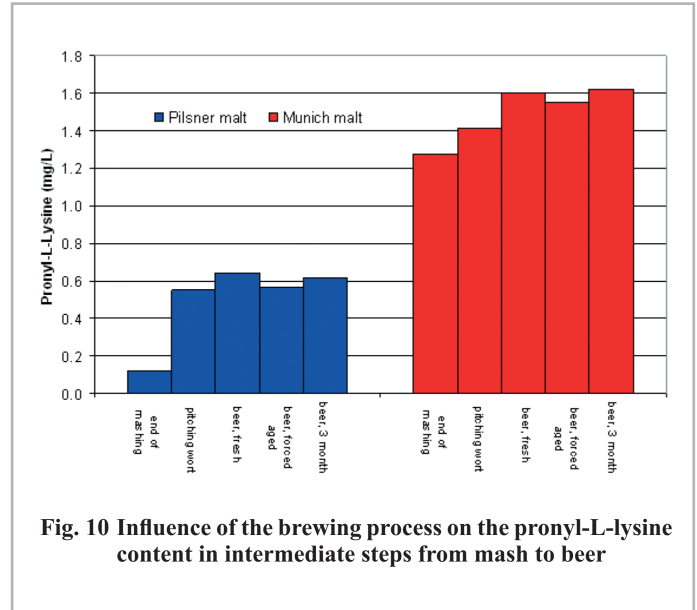

Fig. 10 Influence of the brewing process on the pronyl-L-lysine content in intermediate steps from mash to beer

Table 1 Steps of the brewing process

ratio mash liquor:last runnings	1:1
boiling	70 minutes, atmospheric
fermentation temperature	8 °C
storage time	3 weeks
filtration	sheet filter (80 µm pore size)
filling station	long-tube with CO_2-flushing and pre-evacuation

Table 2 Influence of the germination time on the concentrations of fermentable carbohydrates in malt sugar

sugar / days	Fructose	Glucose	Sucrose	Maltose	Maltotriose	Fermentable sugars
0.5	0.3	3.2	1.0	40.3	5.4	50.2
3	0.5	5.5	1.5	42.9	7.0	57.4
5	1.0	7.3	2.5	41.7	8.8	61.3
7	0.6	7.7	2.6	40.6	7.7	59.2

Table 3 Colours of single malts and malt mixtures

	Colour	Boiled wort colour	Beer colour
Pilsner malt (pi)	3.7	6.3	6.1
Munich malt (mu)	17	23	26
Pale caramel malt (pc)	26	33	-
71.2 % pi + 28.8 % mu	7.6	11	11
100 % Vienna malt	7.2	11	11
81.9 % pi + 18.1 % pc	8.6	12	13