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Interaction effects of fermentation time and added asparagine and glycine on acrylamide content in yeast-leavened bread

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ABSTRACT

The interaction effects of fermentation time and added asparagine and glycine on acrylamide precursors (asparagine and reducing sugars) in dough and content of acrylamide in yeast-leavened wheat bread were studied. Two experiments, with low and high levels of added asparagine (0–0.044 and 0.071–0.476 g/100 g flour, respectively), were performed. Glycine was added (0.042–0.380 g/100 g flour) only in the high asparagine addition experiment. The fermentation time, which was varied between 13 and 164 min, showed a reducing effect on acrylamide precursors in the dough in both experiments ($p < 0.001$). These effects of fermentation were more pronounced in the experiment with low asparagine levels, which resembled levels in ingredients. In contrast, fermentation time did not affect the content of glycine in the dough. Added asparagine increased the levels of asparagine in dough and of acrylamide in bread ($p < 0.001$). A strong correlation was found between the contents of asparagine in the fermented dough and acrylamide in breads at all levels of asparagine. Glycine significantly increased the colour intensity and reduced the acrylamide in bread ($p < 0.001$) with the latter effect being dependent on the level of asparagine.

1. Introduction

Studies aiming to explain the formation of acrylamide (AA) and to provide measures that control its reduction in foods are of crucial importance since AA is a probable human carcinogen (Hogervorst, Schouten, Konings, Goldbohm, & van den Brandt, 2007; IARC, 1994; Olesen et al., 2008). The present knowledge of the chemistry of AA, its formation, analysis, metabolism and strategies for its reduction in food products, are given in inclusive reviews (Claus, Carle, & Schieber, 2008; Friedman, 2003; Lingnert et al., 2002; Stadler & Scholz, 2004; Taeymans et al., 2004; Wenzl, de la Calle, & Anklam, 2003; Zhang, Zhang, & Zhang, 2005). The major pathway for AA formation in food is via the Maillard reaction with different intermediates being involved (Granvogl, Jezussek, Koehler, & Schieberle, 2004; Granvogl & Schieberle, 2006; Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Zyzak et al., 2003). Another pathway for AA formation was suggested to originate from wheat gluten (Claus, Weisz, Schieber, & Carle, 2006). It is now established that the main precursors of AA in cereal products are asparagine (Asn) and reducing sugars with Asn being the limiting precursor (Amrein, Schönbächler, Escher, & Amado, 2004; Becalski, Lau, Lewis, & Seaman, 2003; Mustafa, Andersson, Rosén, Kamal-Eldin, & Åman, 2005; Surdyk, Rosén, Andersson, & Åman, 2004; Yaylayan, Wnorowski, & Locas, 2003).

Factors affecting formation of AA in cereal products have been closely studied regarding different choices of raw material, technology of processing, and reformulation of ingredient composition and usage of additives (Claus et al., 2008). Several measures to reduce AA content in cereal products were suggested with a wide range of reduction levels (Konings, Ashby, Hamlet, & Thompson, 2007). However, secondary effects on the final products were observed in some trials, depending on the treatment. These measures include replacing reducing sugars with sucrose because it lacks the reactive carbonyl group (Amrein et al., 2004). However, this replacement resulted in products that were insufficiently browned and needed colour enhancement. Yeast fermentation was also suggested for AA reduction, where up to 96% of Asn was decreased during extensive fermentation for 6 h (Fredriksson, Tallving, Rosén, & Åman, 2004). A third measure to reduce the content of AA is the addition of glycine (Gly), either by adding it before fermentation or to the surface of the fermented dough before baking (Bråthen, Kita, Knutsen, & Wicklund, 2005; Fink, Andersson, Rosén, & Åman, 2006). The aim of this study was to investigate the interaction effects between fermentation time and added Gly on the reduction of AA content in yeast-leavened soft wheat bread. This interaction effect, to our knowledge, has not been previously presented. This study aims to further identify the levels of Asn where fermentation time or added Gly are more efficient in reducing AA content and

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verify the efficiency of Gly for improving colour intensity. This knowledge is of importance since it provides alternatives that improve reduction of AA in bread.

2. Materials and methods

2.1. Model studies of effect of fermentation time, and added Asn and Gly

Effects of fermentation time, and added Asn and Gly were investigated in two experiments with low and high Asn addition. Levels of factors were chosen such that the final product quality resembles bread made from a standard test baking recipe (Surdyk et al., 2004). All breads were baked at 270 °C for 15 min.

In the low Asn addition experiment, effects of fermentation time were studied in a two-dimensional central composite design with Asn and fermentation time as variable factors (Table 1). In this design, the cube points were run in duplicates, the central point was repeated five times, and the satellite points were performed once. Levels of added Asn ranged from 0 to 0.044 g/100 g flour. The total fermentation times ranged from 13 to 164 min.

In the high Asn addition experiment, effects of added Asn and Gly and fermentation time were studied in a three-dimensional central composite design with Asn, Gly and fermentation time as variable factors (Table 1). The central point was repeated three times and all other points were performed once. In this experiment, the amounts of added Asn ranged from 0.071 to 0.476 g/100 g flour and Gly 0.042 to 0.380 g/100 g flour. The total fermentation times ranged from 13 to 164 min.

Effect of added Gly on AA formation at a low level of added Asn (0.025 g/100 g flour) was further studied in a small scale experiment with three levels of added Gly, 0.042, 0.126 and 0.380 g/100 g flour, and total fermentation time of 67 min.

2.2. Baking procedure

The recipe for baking yeast-leavened wheat bread was adopted from Surdyk et al. (2004). Ingredients were wheat flour (Nordmills, Uppsala, Sweden), dry yeast (Kronjäst Original, Jästbolaget, Sollentuna, Sweden), salt and warm tap water. Flour and yeast were mixed in a farinograph (Brabender, Duisburg, Germany). Solutions of the salt and amino acids, L-asparagine monohydrate and glycine (Merck, Darmstadt, Germany), were dissolved in parts of the water and added to the mixture of yeast and flour; finally the remaining part of water was added. All ingredients were mixed and left to leaven twice according to the designed fermentation time. The ratios between the first and second fermentation times are fixed where the first fermentation time is 17% of the total time. The dough was then divided into portions of 100 g each, kneaded and put into pre-oiled baking tins that were used for a second

Table 1
Central composite design for evaluating the effect of added asparagine, glycine, and fermentation time on the contents of reducing sugars, asparagine and glycine in fermented dough and colour and acrylamide in yeast-leavened wheat bread

Model parameters		Fermentation time (min)	Dough			Bread	
Added asparagine (g/100 g flour)	Added glycine (g/100 g flour)		Reducing sugars (g glucose equivalents/100 g dry dough)	Asparagine (g/100 g dry dough)	Glycine (g/100 g dry dough)	Colour a^*-value	Acrylamide (μg/kg dry bread)
Low asparagine addition experiment							
0.000	–	67	1.71	0.004	NA	NA	13
0.009	–	30	2.00	0.013	NA	NA	54
0.009	–	30	2.30	0.002	NA	NA	41
0.009	–	120	0.97	0.002	NA	NA	19
0.009	–	120	1.35	0.007	NA	NA	34
0.022	–	13	2.41	0.036	NA	NA	66
0.022	–	67	1.64	0.015	NA	NA	42
0.022	–	67	1.58	0.012	NA	NA	51
0.022	–	67	1.64	0.017	NA	NA	44
0.022	–	67	1.62	0.016	NA	NA	50
0.022	–	67	1.64	0.015	NA	NA	48
0.022	–	164	0.63	0.007	NA	NA	23
0.035	–	30	2.30	0.040	NA	NA	98
0.035	–	30	2.21	0.037	NA	NA	82
0.035	–	120	0.92	0.024	NA	NA	67
0.035	–	120	0.88	0.016	NA	NA	35
0.044	–	67	1.53	0.035	NA	NA	141
High asparagine addition experiment							
0.071	0.126	67	1.87	0.07	0.12	2.4	219
0.105	0.065	30	2.38	0.14	0.06	1.6	348
0.105	0.065	120	0.85	0.07	0.06	1.7	279
0.105	0.243	30	2.20	0.12	0.23	2.4	237
0.105	0.243	120	1.06	0.10	0.23	2.2	220
0.184	0.042	67	1.58	0.18	0.04	1.8	657
0.184	0.126	13	2.36	0.23	0.12	2.1	518
0.184	0.126	67	1.87	0.20	0.12	2.3	525
0.184	0.126	67	1.49	0.20	0.11	2.6	683
0.184	0.126	67	1.53	0.19	0.12	2.3	606
0.184	0.126	164	0.77	0.15	0.12	1.5	497
0.184	0.380	67	1.67	0.20	0.35	3.0	378
0.324	0.065	30	2.11	0.41	0.07	1.8	1134
0.324	0.065	120	0.99	0.37	0.06	2.1	1236
0.324	0.243	30	2.25	0.41	0.23	2.7	875
0.324	0.243	120	1.06	0.36	0.24	2.1	869
0.476	0.126	67	1.62	0.56	0.12	2.6	1636

More positive a^*-value indicates more red colour of bread, NA = not analyzed.

fermentation period. A portion of the dough was stored at −20 °C directly after the second fermentation, prior to analysis. Leavened portions were baked for 15 min at 270 °C. Breads were frozen after 1 h of cooling. Frozen dough and bread samples were freeze-dried, crushed and milled in an ultra centrifuge mill (Retsch, Haan, Germany) to pass a 0.5 mm screen for further analysis. The contents of free Asn and Gly in the flour used for baking were in the order of 0.008 and 0.001 g /100 g flour, respectively.

2.3. Analysis of reducing sugars in dough

Reducing sugars in the fermented dough were analysed according to the method described earlier (Fink et al., 2006; Hostettler, Borel, & Deuel, 1951). Samples were extracted with water and then mixed with Sumner's reagent containing 1% (w/v) 3,5-dinitrosalicylic acid (Merck, Darmstadt, Germany). The solution was heated in a water bath (90 °C), cooled and diluted. The absorbance was measured in a spectrophotometer (Shimadzu, Kyoto, Japan) at 530 nm. All samples were analysed in triplicate. Results are presented as glucose equivalents in g/100 g flour.

2.4. Analysis of amino acids in dough

Analyses of Asn and Gly were performed in fermented dough according to the method described by Mustafa, Åman, Andersson, and Kamal-Eldin (2007). Freeze-dried homogenized samples were extracted in 50% alcohol and an aliquot of the extract was subjected to solid phase extraction (SPE) and derivatization steps using the EZ-faast technology (Phenomenex, 2001). The derivatized amino acids were analysed using GC-FID.

2.5. Analysis of physical parameters of bread

Once the bread was out of the oven, the central crumb temperature was measured using a thermocouple digital thermometer. Fresh weight, volume by displacement with sagosand and porosity, according to the Dallman scale, were recorded after 1 h of cooling at room temperature according to Surdyk et al. (2004). Colour was measured on milled breads with a Chroma Meter (Minolta, Osaka, Japan) as the a^* value (positive red, negative green). Measurements were performed three times on each sample that was thoroughly mixed in-between measurements.

2.6. Extraction and analysis of AA in bread

Extraction and analysis of AA were performed according to the procedure described before (Petersson, Rosén, Turner, Danielsson, & Hellenäs, 2006; Rosén, Nyman, & Hellenäs, 2007). Freeze-dried samples were extracted with water after the addition of deuterium-labelled AA as internal standard. Extracts were centrifuged and the supernatant was exposed to solid phase extraction for clean-up. The determination of AA content in the extracts was done using liquid chromatography-tandem mass spectrometry. Results of AA analysis are given in dry bread.

2.7. Statistics

The baking experiments were designed and evaluated by regression analysis using the software Minitab (Minitab Inc., State Collage, PA, USA).

3. Results and discussion

3.1. General

Two experiments, with low and high levels of added Asn, were designed to study the interaction effects of fermentation time and added Asn and Gly on AA precursors (Asn and reducing sugars) in the dough and the reduction of AA content in bread made from sifted wheat flour. The sifted fraction from wheat grain is known to have the lowest content of Asn (Mustafa et al., 2007). The reducing effect of added Gly on AA content was only tested in

Table 2
P-values and regression coefficients (in parentheses) for the effects of (a) added asparagine, and fermentation time and their interactions, (b) added asparagine, glycine, fermentation time, and their interactions, on asparagine, glycine and reducing sugars in fermented dough and degree of redness (a^* value) and acrylamide content in bread[a]

Factors	Dough			Bread	
	Reducing sugars	Asparagine	Glycine	Colour	Acrylamide
Low asparagine addition experiment					
Asparagine (A)	NS	<0.001 (0.011)	NA	NA	<0.001 (25.6)
Fermentation time (B)	<0.001 (−0.563)	<0.001 (−0.007)	NA	NA	0.005 (−14.1)
A × A	NS	NS	NA	NA	0.038 (10.1)
B × B	NS	NS	NA	NA	NS
A × B	0.041 (−0.092)	0.020 (−0.004)	NA	NA	NS
R^2 adjusted (%)	96	90	NA	NA	77
High asparagine addition experiment					
Asparagine (A)	NS	<0.001 (0.14)	NS	NS	<0.001 (396)
Glycine (C)	NS	NS	<0.001 (0.088)	0.001 (0.31)	0.001 (−93)
Fermentation time (B)	<0.001 (−0.559)	<0.001 (−0.02)	NS	NS	NS
A × A	NS	<0.001 (0.04)	NS	NS	<0.001 (113)
C × C	NS	NS	<0.001 (0.028)	NS	NS
B × B	NS	NS	NS	0.004 (0.25)	NS
A × C	NS	NS	NS	NS	0.025 (−57)
A × B	NS	NS	NS	NS	NS
C × B	NS	NS	NS	NS	NS
R^2 adjusted (%)	90	99	100	78	98

NA = not analyzed.
NS = not significant.
Explained variances by the model are given as adjusted R^2, in percent[b].
[a] For the experimental design and response model refer to Table 1.
[b] Coefficients are based on coded levels of the design.

the experiment with high addition of Asn and Gly as it was previously shown that this effect of Gly is better perceived in systems where there are high contents of Asn (Fink et al., 2006).

3.2. Effects on reducing sugars, asparagine and glycine in dough

The contents of reducing sugars in the fermented dough gave similar variation in both low and high Asn addition experiments, where they ranged from 0.63 to 2.41 and 0.77 to 2.38 glucose equivalents/100 g flour, respectively (Table 1). Reducing sugars were decreased significantly ($p < 0.001$) by fermentation time (Table 2). Surface plots further illustrate the important effect of fermentation time in both experiments (Fig. 1). Asn, as individual factor, had no significant effect on reducing sugars in the low Asn addition experiment. However, when evaluating its interaction with fermentation time, it showed a small but significant effect on the content of reducing sugars. This indicates that the lowering effect of fermentation time on the content of reducing sugars is dependent on the level of Asn in the system. Yeast is reported to consume Asn during fermentation for its metabolic activity (Benedito De Barber, Prieto, & Collar, 1989). The content of Asn present in this system might have facilitated metabolic activity and consequently led to increased consumption of sugars during fermentation. This interaction was not observed in the high Asn addition experiment, probably because the levels of Asn were not limiting for the yeast metabolic activity. A strong and consistent correlation was, however, found between the content of reducing sugars in the dough and fermentation time, regardless of the level of Asn present (Fig. 2).

The content of Asn in the fermented dough was affected differently in the two experiments. In the low Asn addition experiment, the content of free Asn varied from 0.002 to 0.040 g/100 g dry dough while, in the high Asn addition experiment, the variation was in the range 0.07–0.56 g/100 g dry dough (Table 1). In both experiments, the content of Asn in fermented dough increased significantly by Asn addition ($p < 0.001$) and was decreased significantly by long fermentation time ($p < 0.001$) (Table 2). In the low Asn addition experiment, the decreasing effect of fermentation time on Asn content in the fermented dough was higher with increasing levels of added Asn (Fig. 3A). However, this interaction

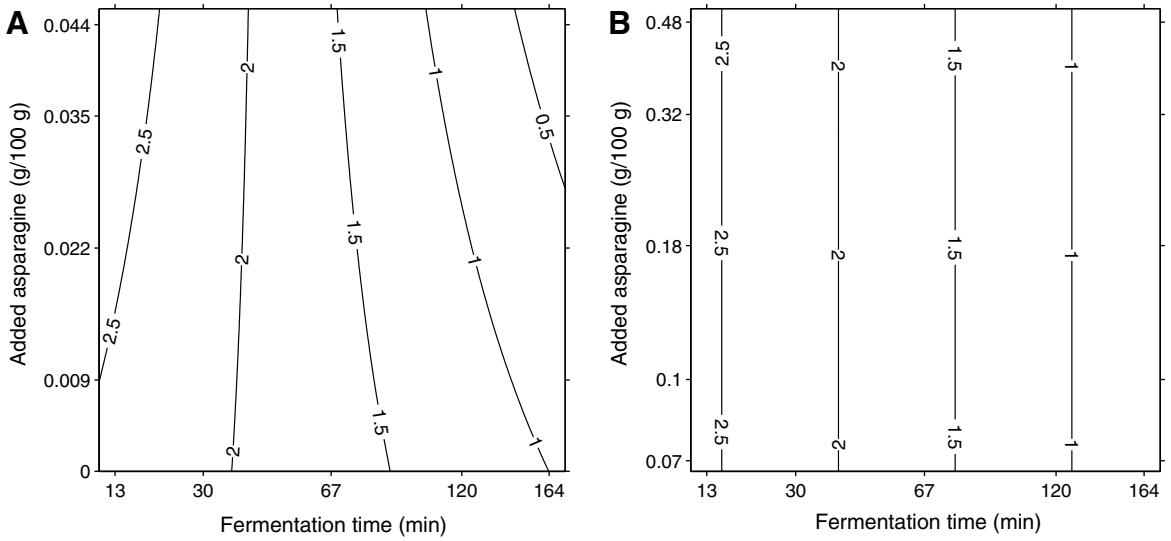

Fig. 1. Effect of asparagine addition and fermentation time on reducing sugars content (g glucose equivalents/g dry dough) in fermented dough; (A) low Asn addition experiment, (B) high Asn addition experiment.

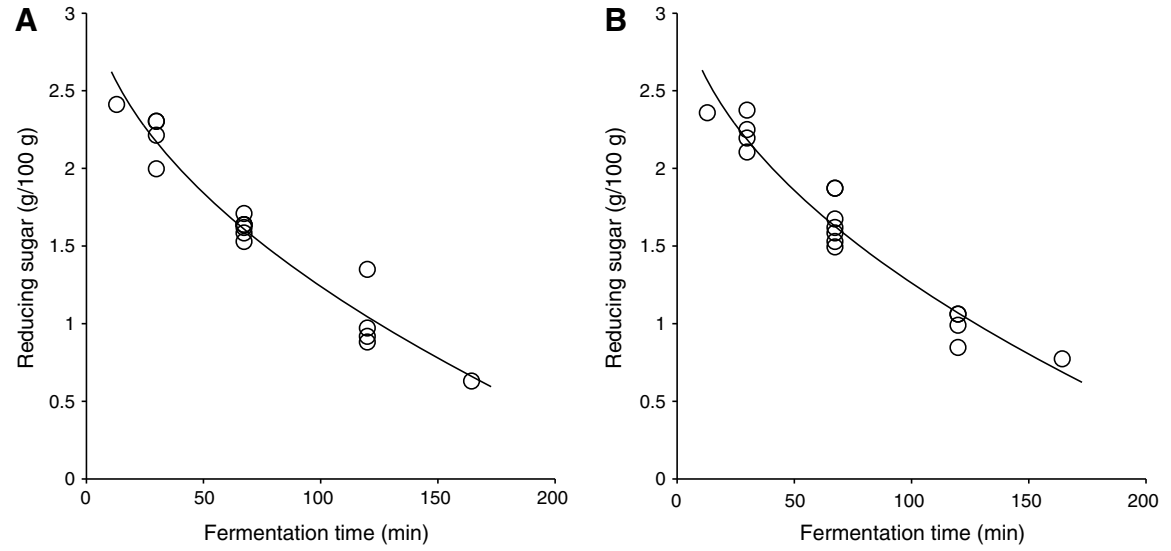

Fig. 2. Reducing sugars (g glucose equivalents/g dry dough) and fermentation time for experiments with low (A) and high (B) addition of asparagine. The lines show the regression models from Fig. 1.

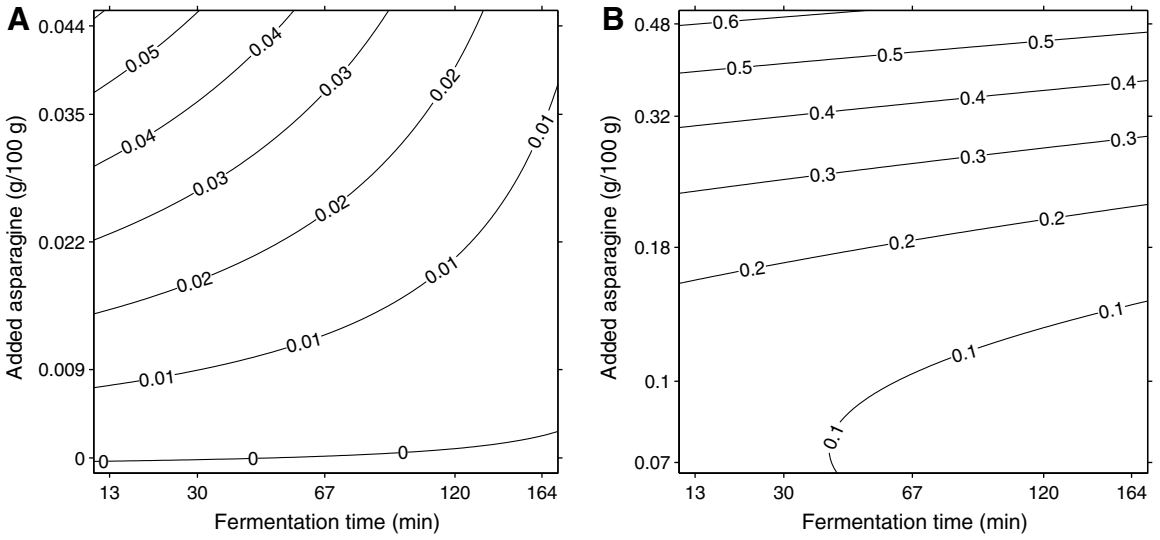

Fig. 3. Effect of added Asn and fermentation time on Asn content (g/100 g dry dough) in fermented dough; (A) low Asn addition experiment, (B) high Asn addition experiment.

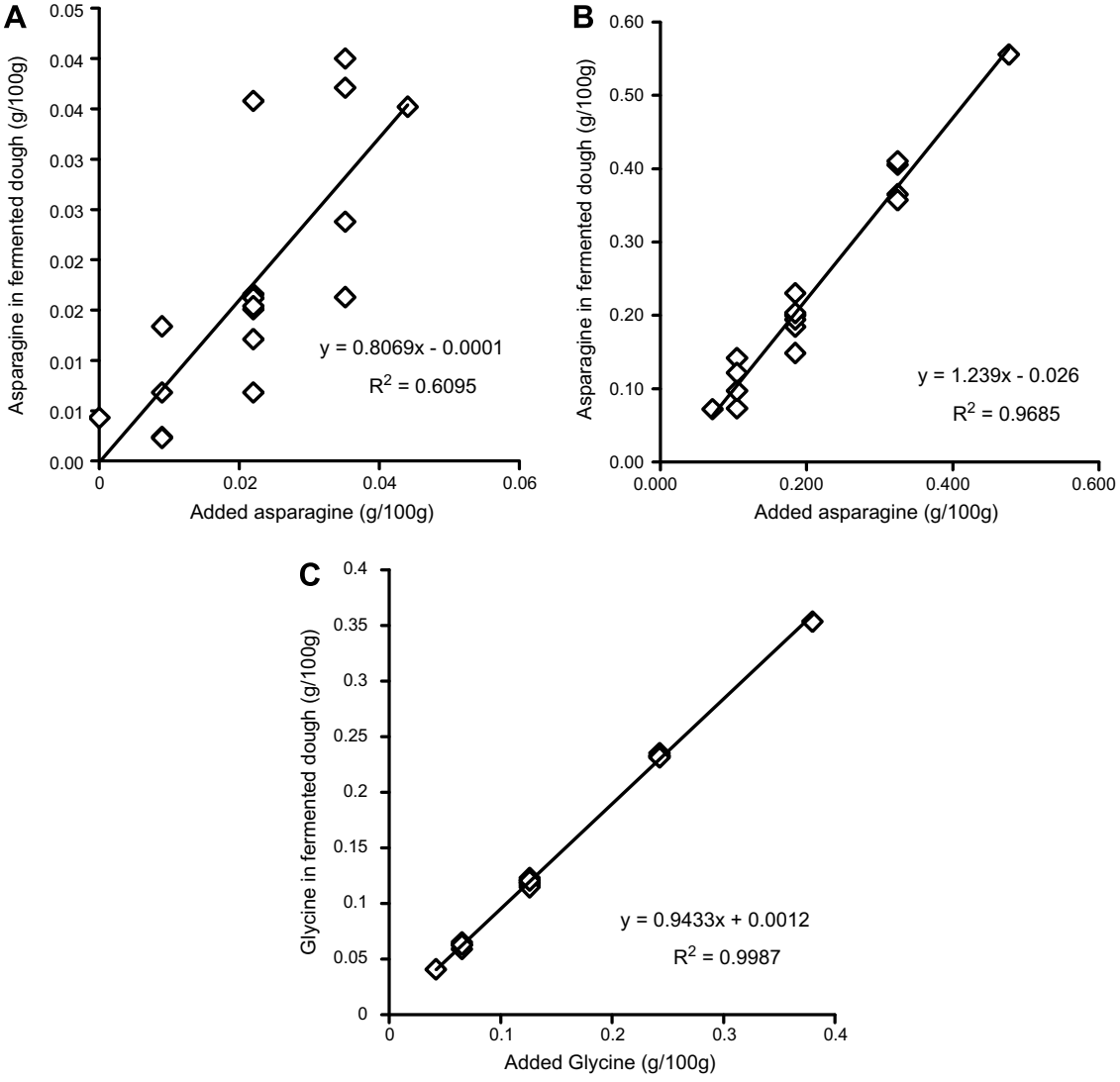

Fig. 4. Correlation between added amino acids and their contents in fermented dough; (A) Asn in the low Asn addition experiment, (B) Asn in the high Asn addition experiment, (C) Gly in the high Asn addition experiment.

between fermentation time and Asn was not observed in the high Asn addition experiment (Table 2 and Fig. 3B). The model describing Asn content in the dough was able to explain 99% of the variation in the high Asn addition experiment and 90% in the low Asn addition experiment. Correlation coefficients between added amino acids and their contents in the fermented dough were high for Asn in the high Asn addition experiment ($R^2 = 0.97$) but low for the low Asn addition experiment ($R^2 = 0.61$) (Fig. 4). This difference in correlation is because of the different effects of time. The effect of long fermentation on Asn content of dough complies with earlier results showing a substantial decrease in Asn in dough made from fractions with high Asn levels (Fredriksson et al., 2004).

Gly, measured in the dough of high Asn addition, was found to range from 0.04 to 0.35 g/100 g dry dough, a content that is similar to that added to the flour and that was affected by neither added Asn nor fermentation time. This was further supported with a strong correlation between added Gly and its content in fermented dough ($R^2 = 1.0$), showing that Gly is not consumed during fermentation. To our knowledge, this is the first study that presents the effect of fermentation time on Gly content during bread baking.

3.3. Effects on physical parameters of bread

Physical parameters of breads were affected by fermentation time. At longer fermentation time, there was an increase in bread volume but a reduced fresh weight, central crumb temperature, and porosity index ($p < 0.001$). Breads baked with long fermentation times had larger pores (lower Dallman index, $p < 0.001$) due to the enhanced gas production by the yeast. In accordance, central crumb temperature was found to be lower after longer fermentation times ($p < 0.001$).

Effect on colour was only investigated in the experiment with added Gly since it was shown previously that added Gly, but not Asn, contributes to colour formation (Fink et al., 2006; Mustafa et al., 2005). When testing the effect of added Asn, added Gly and fermentation time on colour formation, the degree of redness (a^* value) ranged from 1.5 to 3. This variation was significantly, ($p < 0.001$) affected by added Gly and the quadratic factor of fermentation time, which indicated a nonlinear effect (Tables 1 and 2). The surface plot (Fig. 5) shows an increase in the intensity of the red colour with Gly addition and fermentation time, where

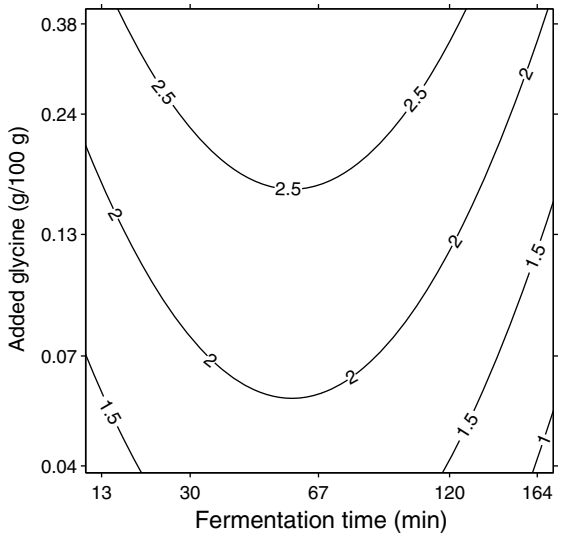

Fig. 5. Surface plot illustrating the effects of added Gly and fermentation time on degree of redness (a^* value).

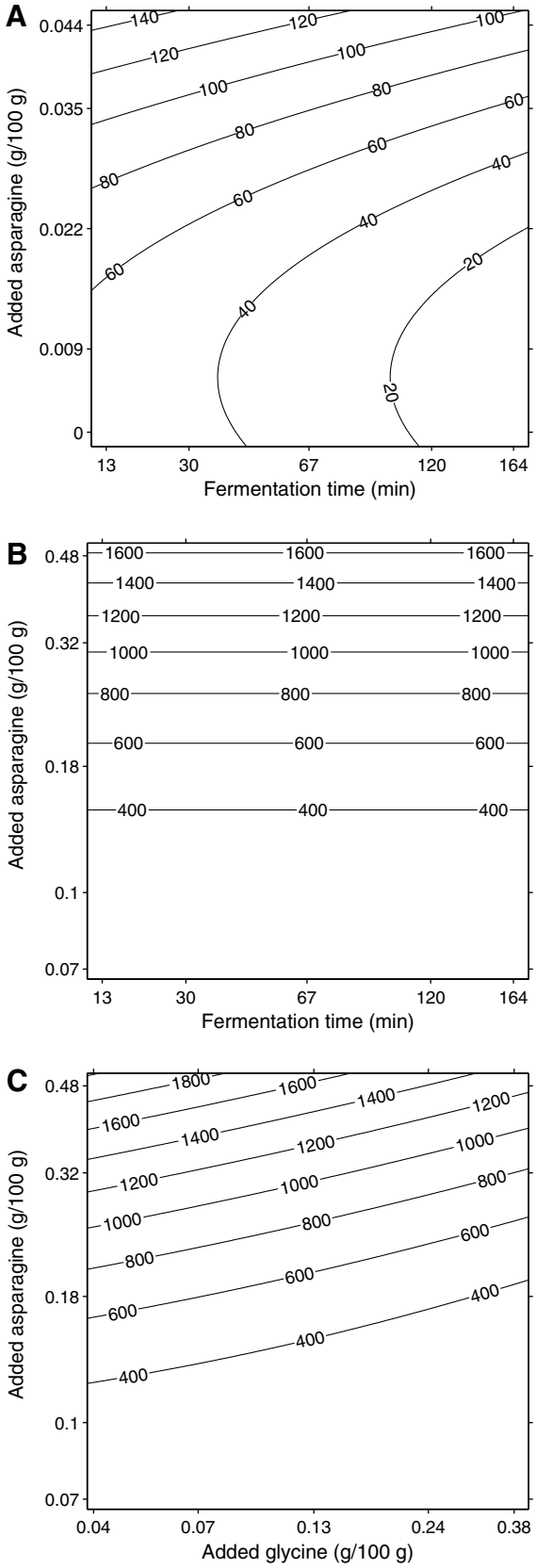

Fig. 6. Surface plots illustrating acrylamide content (μg/kg dry bread); (A) as affected by added Asn and fermentation time in the low Asn addition experiment, (B) as affected by added asparagine and fermentation time in the high Asn addition experiment, (C) as affected by added Asn and Gly in the high Asn addition experiment.

the highest colour intensity is achieved at intermediate fermentation times. The significant effect of added Gly indicates that, unlike Asn, Gly is taking part in the Maillard reaction(s) leading to colour formation. It was previously reported that Gly, when heated with reducing sugars, resulted in high browning intensity (Ashoor & Zent, 1984). The decrease in colour at longer fermentation times might be due to the consumption of other amino acids and sugars, i.e. other main precursors for colour formation. The model explains 78% of the variation, indicating that most of the factors affecting colour formation in this system are covered by the model.

3.4. Effects on acrylamide content in bread

The content of AA in bread varied from 13 to 141 μg /kg and 219 to 1636 μg/kg in the low and high Asn addition experiments, respectively. In both experiments, added Asn had a high increasing effect on AA ($p < 0.001$) (Tables 1 and 2). Fermentation time had a reducing effect on AA formation in the low Asn addition experiment. The surface plot (Fig. 6A) shows that the highest level of formed AA coincides with an increasing addition of Asn and a short fermentation time. The effect of added Asn on the formation of AA was higher than the decreasing effect of fermentation time. The direct relationship between Asn content and AA formation is in line with previous results showing that the Maillard reaction is the major pathway that results in AA formation (Yaylayan & Stadler, 2005; Zyzak et al., 2003). A similar decreasing effect of fermentation time on the content of AA was not observed in the high Asn addition experiment (Fig. 6B). It seems that Asn was in such abundance that even a long fermentation time did not affect its contribution to AA formation. When the effect of fermentation time on AA formation was studied in bread made from two different recipes and Asn contents, a decrease in AA was found to range from 77 to 87%, depending on the recipe (Fredriksson et al., 2004). In the present study, the amount of Asn consumed by yeast plays an insignificant role when ample amounts were present in the high Asn addition experiment. There was a strong correlation between Asn content in dough and AA content in bread at all levels of added Asn (Fig. 7), which further supports the finding that Asn is the critical precursor for AA formation in bread (Amrein et al., 2004; Mustafa et al., 2005; Surdyk et al., 2004).

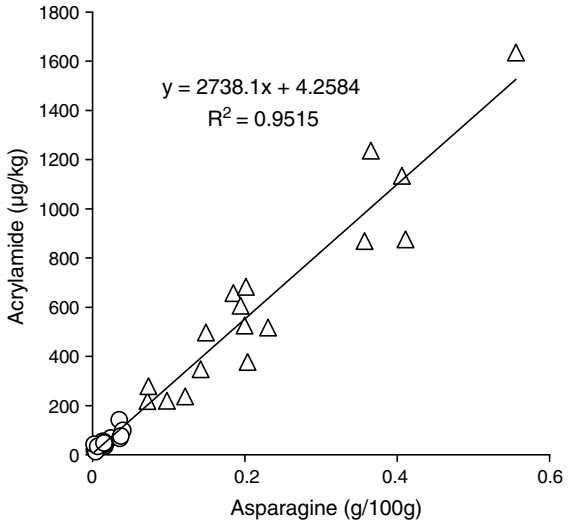

Fig. 7. Correlation between Asn content in fermented dough and acrylamide content in bread in [○] the low Asn addition experiment and [Δ] the high Asn addition experiment.

It was previously proposed that added Gly would lead to a decrease in AA at higher levels of Asn (Fink et al., 2006). In this experiment, added Gly and its interaction with Asn had a low but significant effect on the reduction of AA content. AA content increased with the addition of Asn and the reducing effect of added Gly was higher at increased levels of added Asn (Fig. 6C). The negative interaction effect between added Asn and Gly indicates that the decreasing effect of Gly on AA is dependent on the level of added Asn, possibly due to competition with Asn for reducing sugars and/or to reaction(s) with formed AA (Rydberg et al., 2003). It was proposed that a Michael addition reaction takes place with the amino group of Gly, resulting in AA binding (Wedzicha, Mottram, Elmore, Koutsidis, & Dodson, 2005).

The effect of added Gly on formed AA was further studied in a smaller experiment with a low level of added Asn (0.025 g/100 g) and three different levels of added Gly. There was no effect of added Gly on the amount of AA formed (results not shown), indicating that, in this system, added Gly did not affect AA formation. Thus, the ability of Gly to reduce AA is only pronounced when the content of Asn present in the system is high. It was reported earlier that the reducing effect of Gly is high when it is present in high amounts (Bråthen et al., 2005; Low et al., 2006).

4. Conclusions

Asn content in sifted wheat flour is in the order of 0.008 μg/100 g (Mustafa et al., 2007) and the content of AA in soft wheat bread is in the range <30–160 μg/kg fresh bread (Svensson et al., 2003). In this study, we have shown that yeast fermentation has an important role in controlling the formation of AA at low levels of Asn, such as those generally present in bread raw materials. Nevertheless, extensive yeast fermentation is not recommended since it results in degradation of the protein network and subsequent flattening of bread rolls and/or production of monochloropropanediol isomers (MCPDs), known as potential genotoxic carcinogens (Fredriksson et al., 2004; Hamlet, Sadd, & Gray, 2004).

Furthermore, fermentation time has a significant reducing effect on the level of reducing sugars in dough but this effect was not translated to an effect on AA level in bread, supporting the previous finding that Asn is the critical precursor for AA formation in cereal products. Asn level in dough is highly correlated with the level of AA in bread (Fig. 7). The participation of precursors other than Asn is more pronounced at low Asn levels in dough. For example, gluten has also been suggested as a precursor for the formation of AA in cereal products (Claus et al., 2006).

Gly, which is not consumed during fermentation, was found to have a small but significant decreasing effect on AA levels in bread when baked with dough containing high levels of Asn. Our research suggests that, even if Gly has no pronounced effect in reducing AA, its addition during bread baking has the merit of increasing the intensity of the red colour. This is of practical application for AA controlling measures, leading to reduced colour intensity, e.g. reducing temperatures or times of baking or replacing reducing sugars with sucrose. This study provides some practical knowledge pertinent to the reduction of AA in yeast-fermented wheat bread. Reduction can also be achieved by other means, including controlling the content of Asn in the ingredients, the choice of raw materials, and the use of newly developed asparaginase.

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