Research Article

Suppressed acrylamide formation during baking in yeast-leavened bread based on added asparaginase, baking time and temperature using response surface methodology

Mashaer Matouri, Iran Alemzadeh *

Department of Chemical and Petroleum Engineering, Sharif University of Technology, Tehran, Iran.

Abstract

Background and Objective: Acrylamide as a toxic substance for human beings is produced by Maillard reaction at high temperatures. In this research, this reaction can be inhibited based on using aspariganse enzyme, controlling the cooking time and temperature during baking in yeast-leavened bread.

Material and Methods: In this study, a response surface methodology 5-level-3-factor central composite design was applied to study the effects of asparaginase (300-900 U Kg^{-1} of flour), baking temperature (230-280°C) and baking time (13-16 min) on acrylamide formation in yeast-leavened wheat bread.

Results and Conclusion: Added asparaginase showed a reducing effect on acrylamide formation ($p \leq 0.0001$). Baking temperature significantly increased the acrylamide content in bread ($p \leq 0.0001$). A strong correlation was found between the baking temperature and acrylamide formation. Baking time and its interaction with asparaginase had a low but significant reducing effect on acrylamide content in bread ($p \leq 0.0001$). Three parameters of the cooking temperature and time as well as enzyme concentration have been optimized using response surface methodology, their values obtained 245.71°C, 14.55 min and 752.15 U Kg^{-1}, respectively. Enzymatic process could be suggested as a safe and convenient method for preventing acrylamide formation in bread making.

Conflict of interest: The authors declare no conflict of interest.

1. Introduction

Acrylamide is a toxicant and defined as a suspected human carcinogen by the International Agency for Research on Cancer [1]. In 2002, a research group showed that acrylamide is found in a range of cooked foods, with the highest content range in carbohydrate-rich foods, 50-4000 μg Kg^{-1}, and a range of 5-50 μg Kg^{-1} in protein-rich foods [2].

Following these events, the Joint FAO/WHO Expert Committee on Food Additives undertook a comprehensive review of data on the occurrence of acrylamide during baking in starchy foodstuffs which are mostly fried, roasted or baked from many countries, mainly in Europe and North America. The outcome of this review was that the main acrylamide containing food groups were identified as potato-based products, cereal-based products and coffee [3].

The mutagenic and carcinogenic properties of acrylamide are related to its metabolite glycidamide.

Glycidamide has the ability to form DNA adducts, which account for a genotoxic and cancer risk increasing agent [4]. The first studies on the mechanistic pathway for the formation of acrylamide in food proposed the Maillard reaction as the main pathway, which is a reaction between asparagine and reducing sugar [5,6]. This reaction mainly occurs at high temperatures. In the previous study [7], potato crisps which were fried at high temperatures showed the significant amounts of acrylamide. Many studies have investigated factors such as time, temperature and minerals affecting acrylamide formation in foods and means to introduce measures for acrylamide formation in food [8-12].

A possible way to reduce acrylamide formation is the control of time and temperature. The effect of time and baking temperature in cereal-based products has been investigated in numerous studies [13-15]. Lower frying time and temperature have been shown to lead to

acrylamide reduction [16]. Recently, it has been shown that reducing the frying temperature from 180 to 165°C and to 150°C, decreased acrylamide content in fried potatoes by 6% and 18%, respectively [17]. Another study showed that the content of acrylamide in wheat bread linearly increased with time and baking temperature [1].

Addition of the enzyme asparaginase, obtained from *Aspergillus (A.) oryzae* and *A. niger* has been reported as a method to reduce the formation of acrylamide since it results in the hydrolysis of asparagine to aspartic acid and ammonia [18,19]. In an experiment with Gingerbread, addition of asparaginase resulted in a 75% decrease in asparagine content, which was further resulted in 55% reduction of acrylamide content [13]. Recently the effective dose for asparaginase was reported to be 200-1000 μg Kg^{-1} dough [20]. The effect of asparaginase in reducing acrylamide content seems to vary according to the product type and formulation parameters. Amrein reported that addition of asparaginase has no effect on acrylamide formation in hazelnut biscuit [20]. However, a pretreatment of potato slices with asparaginase before frying reduced acrylamide formation [18]. Therefore a general conclusion from the experiments using aspara-ginase suggests that this method is quite promising for the reduction of acrylamide while has no inconvenient effect on the organoleptic properties of cooked products [17,18,21].

The major goal of this study was to investigate the effects of different asparaginase amounts, baking time and baking temperature on the reduction of acrylamide content in yeast-leavened wheat bread. In addition, this study aimed to determine the optimal combination condition of these three parameters for minimizing acrylamide content. Therefore, response surface methodology 5-level-3-factor central composite design was assigned and applied in this study (Design-Expert version7.1). Investigation on the interaction between three parameters and determination of the optimal quantity for each parameter to prevent acrylamide formation is the novelty of this research study.

2. Materials and Methods

2.1. Baking procedure

The yeast-leavended wheat bread was baked according to the method used by Surdyk [15]. The ingredients were wheat flour, dry yeast (Fariman, Iran Mellas Company, Iran), salt and warm tap water. Dry yeast, salt and warm tap water were added to the recipe at 2%, 1.2% and 55% flour weight, respectively. Different levels of asparaginase (Elspar, Lundbeck, Deerfield, IL, USA, 10000U), in the range from 300 U Kg^{-1} of flour to 900 U Kg^{-1} of flour, were added to this recipe according to central composite design (Table 1). Flour and yeast were mixed in a farinograph (Even Baker, China) at low speed for 2 min. To assure a homogeneous distribution in the dough, asparaginase was dispersed in the aqueous phase before being added to the dry ingredients. Solutions of the salt and asparaginase were dissolved in parts of the water and added to the mixture of yeast and flour, and finally the remainder of the water was added. All ingredients were mixed and left to leaven for 15 min, dough was then divided into portions of 100 g each. Thereafter, dough was molded and placed in pre-oiled baking tins that were taken for a second fermentation period for 55 min. Leavened portions were baked at different baking temperatures and times according to central composite design (Table 1). In particular, the baking temperature ranged from 230 to 280°C and the baking time from 13 to 16 min. Breads were dried at room temperature for further analysis, crushed and milled homogeneously in blender (Pars khazar, Iran) to pass a 0.5 mm screen.

2.2. Sample preparation

Finely pulverized sample of 2.0 g was weighed and put into a 50 ml centrifuge tube. 10 ml of Milli-Q water was added and the sample was extracted for 3 min on a tube shaker. Then the suspension was centrifuged at 13584 ×g for 10 min, 5 ml of the supernatant was transferred into another centrifuge tube. Carrez I and II solutions were prepared by dissolving 15 g potassium hexacyanoferrate and 30 g zinc sulfate in 100 ml Milli-Q water [22]. Fifty μl Carrez I and 50 μl Carrez II were added to the centrifuge tube to precipitate proteins from the co-extractives and then followed by centrifugation at 13584 ×g for 10 min. Afterwards, 1 ml of clear supernatant was filtered through a 0.45 μm syringe filter and kept in 1.5 ml amber glass vial at 4°C before analysis. Prior to HPLC-UV analysis, the clean sample extract was further filtered through 0.22 μm PVDF syringe filter [17,23].

2.3. HPLC-UV analysis

The HPLC system used for the analysis equipped with a diode array detector UV-Vis and a S5200 autosampler (Sykam Chromatography, Clarity, Germany). The calibration standards and sample extracts were injected via 20 μl sample onto a 250 mm × 4.6 mm, 5 mm Nucleodur C18ce column (Nucleodur, MACHEREY-NAGEL, Germany) at 22°C. Milli-Q water was employed as mobile phase. The flow rate was 0.6 ml min^{-1}. The detection wavelength was 202 nm [17,21].

Table 1 .Design matrix of central composite design.

Run	Asparaginase Concentration (U Kg⁻¹) (*C*)	Baking Temperature (°C) (*A*)	Baking Time (min) (*B*)	Responses(µg Kg⁻¹) (*R1*)
1	600	255	14.5	550
2	778.38	240.13	15.39	254
3	600	255	14.5	511.98
4	600	255	14.5	511.468
5	600	255	14.5	574.502
6	600	255	16	854.9
7	600	255	14.5	570.28
8	600	255	13	1305
9	900	255	14.5	489
10	600	255	14.5	563.603
11	600	230	14.5	235.68
12	778.38	269.67	15.39	1296
13	421.62	240.13	15.39	381
14	421.62	240.13	13.61	1129.48
15	300	255	14.5	1206
16	600	280	14.5	1623
17	778.38	240.13	13.61	275
18	778.38	269.87	13.61	989.43
19	421.62	269.87	13.61	1804.5
20	421.62	269.87	15.39	1503

2.4. Experimental design for acrylamide reduction in yeast-leavened wheat bread

The baking experiments were generated and evaluated by regression analysis of the experimental data and quadratic model using the Design-Expert® Software (Trial version 7.1, Stat-Ease Inc. Minneapolis, USA). The levels of baking temperature (A), baking time (B) and aspara-ginase (C) were selected for studies using the central composite design. The ranges of variables (Table 2) were decided according to the literature data. A set of 20 experiments was designed (Table 1). The design matrix with different variables was set at five levels (-2, -1, 0, +1, +2).

2.5. Statistical analysis and modeling

Data on reduction of acrylamide content in yeast-leavened wheat bread were subjected to Analysis of Variance (ANOVA) appropriate to the design of the experiments. The mathematical relationship of the inde-pendent variable and the response (acrylamide concen-tration) was calculated by the second order- polynomial regression, which is given by Eq. 1:

Eq. 1

$R1 = ß_0 + ß_1A + ß_2B + ß_3C + ß_{11}A^2 + ß_{22}B^2 + ß_{33}C^2 + ß_{12}AB + ß_{13}AC + ß_{23}BC$

Where *R1* is the predicted response; *A*, *B* and *C* represent the levels of the factors according to Table 1 and $ß_0$, $ß_1$,…, $ß_{23}$ represent coefficient estimates with $ß_0$ having the role of a scaling constant. This equation is used to evaluate the linear, quadratic and interactive effect of independent variables on the selected response. The model was later put to validation by performing the 20 experiments generated by design expert.

3. Results and Discussion

3.1. Fitting the response surface model

The experiments were performed according to the central composite design under the defined conditions and the responses obtained from the experimental runs which are given in Table 1. Using the central composite design, 20 sets of tests with appropriate combinations of baking temperature (*A*), baking time (*B*) and asparaginase concentration (*C*) were conducted. The results in Table1 were fitted to second-order polynomial model by applying regression analysis. As shown in Table 3, the model was highly significant with very low P-value (p≤0.0001). Moreover, the "fitness "of the model was investigated through the lack-of-fit test (p>0.05), which indicated the suitability of model to accurately predict the variation [24,25]. A measure of the model's overall performance referred to as the coefficient of determination and denoted by R^2 must be considered [24,26]. The R^2 was found to be 0.9955. The adjusted R^2 value (0.9915) also indicated the significance of the model. At the same time, the value of the CV was 5.42%, indicating high degree of precision and reliability of the experimental values (Table 3).

Table 2. Coded factors for three independent variables used in central composite design.

Variables	-2	-1	Coded 0	+1	+2
Asparaginase concentration (U Kg^{-1})	300	421.619	600	778.381	900
Baking temperature (°C)	230	183.72	255	269.865	280
Baking time (min)	13	13.6081	14.5	15.3919	16

Table 3. Analysis of variance for the effect of the independent variables on the response and the regression coefficients of fitted quadratic equation obtained from experimental results.

| Source	DF	Acrylamide concentration (µg Kg^{-1})		
		Coefficient	Sum of Squares	p-Value
Model	9	547.71	4.507	≤0.0001
Linear				≤0.0001
ß1	1	431.04	2.537	≤0.0001
ß2	1	-111.40	1.695	≤0.0001
ß3	1	-235	7.542	≤0.0001
Quadratic				≤0.0001
ß11	1	130.38	2.450	≤0.0001
ß22	1	183.63	4.859	≤0.0001
ß33	1	101.44	1.483	≤0.0001
Interaction				≤0.0001
ß12	1	96.82	74990.96	0.0001
ß13	1	-5.07	205.94	0.7567
ß23	1	166.94	2.230	≤0.0001
Residual	10		20306.73	
Lack-of-fit	5		16234.83	0.0776
pure error	5		4071.9	
Total	19		5.527	
R^2		0.9955		
Adj-R^2		0.9915		
CV		5.42		

3.2. Effects on acrylamide content in bread

From Table 1, shows that the concentrations of acrylamide in yeast-leavened wheat bread varied from 235.68 to 1804.5 µg Kg^{-1} dry bread. Added asparaginase enzyme had significant decreasing effects on acrylamide formation (p≤0.0001) (Figure. 1B and C), which further supports the finding that addition of the enzyme asparaginase is a method to reduce the formation of acrylamide since it results in the hydrolysis of the critical precursor (asparagine) for acrylamide formation in bread [13,19]. In an experiment with dried potato powder, asparaginase resulted in a 90-97% decrease in acrylamide content [17]. Similar results were also reported in a model system of wheat [27].

Elevated baking temperatures had a highest increasing effect on acrylamide content (p≤0.0001) (Figure. 1(A) and (B)). The surface plot (Figure. 1B) shows that the minimum level of formed acrylamide coincides with an increasing addition of asparaginase and a lowest baking temperature,

also shows that the effect of baking temperature on the formation of acrylamide was higher than the decreasing effect of added asparaginase. The direct relationship between baking temperature and acrylamide formation was studied in a starch system, of freeze-dried flat bread dough and flat breads, an increase in acrylamide was found at approximately 200°C depending on the system and the baking time [14].

It was previously proposed that acrylamide concen-tration increased by increasing baking time [13]. In this experiment, decreasing baking time and its interaction with added asparaginase had a low but significant effect on the reduction of acrylamide content (p≤0.0001) (Figure. 1A and C). The positive interaction effect between added aspara-ginase and decreased baking time indicates that the decreasing effect of baking time on acrylamide is dependent on the activity of asparaginase, possibly due to decrease enzyme activity during baking process (Figure. 1C).

(A)

(B)

(C)
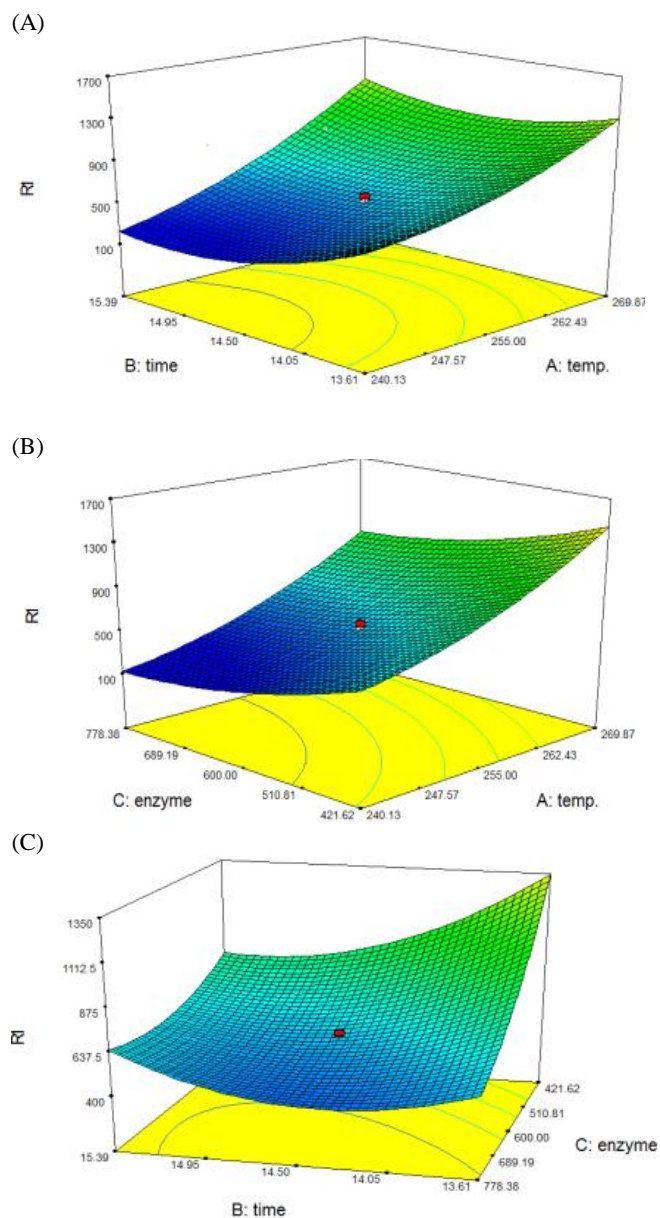
Figure. 1 Response surface plots illustrating acrylamide content (R1), (μg Kg⁻¹ dry bread); (A) as effected by baking time (min) and temperature, (°C); (B) as effected by added enzyme (U Kg⁻¹) and baking temperature (temp.), (°C); (C) as effected by added enzyme (U Kg⁻¹) and baking time (min)

3.3. Optimization and validation of response surface methodology results

The optimal values of the selected variables were obtained by solving the regression equations using Design-Expert software. According to the response surface analysis, the minimum acrylamide content can be achieved at asparaginase concentration of 752.15 μg Kg⁻¹, baking temperature of 245.71°C and baking time of 14.55 min. This optimum condition provides the lowest value of a concentration 204.28 μg Kg⁻¹ (Table 4). In order to validate the optimization results, three additional experiments were carried out at the optimum conditions within the experimental range obtained above (Table 4). No significant difference (p>0.05) was found between the experimental and predicted value, which indicates the high accuracy of the presented model.

4. Conclusion

This study used response surface methodology to determine the effects and interactions between the amount of asparaginase, baking time and temperature on the reduction of acrylamide in yeast-leavened wheat bread. The results showed that asparaginase has an important role in controlling the formation of acrylamide in bread procedure. Temperature increasing shows strong effect on acrylamide formation. Baking time shows low decreasing effect on this acrylamide formation process. The effects of various levels of asparaginase, baking time and temperature were studied by central composite design. The results showed that the second-order polynomial model with high R² (0.9955) can be used to predict the optimal levels of variables for any given conditions within the experimental range. The results verified the previous model that asparaginase 752.15 UKg⁻¹, baking temperature at 245.71°C and baking time at 14.55 min was the optimal combination for obtaining minimum acrylamide concentration in bread.

Table 4. Validation of central composite design using different levels of asparaginase, baking temperature and time.

Run	Asparaginase Concentration (U Kg⁻¹)	Baking Temperature (°C)	Baking Time (min)	Predicted Acrylamide (μg Kg⁻¹)	Experimental Acrylamide (μg Kg⁻¹)
1	752.15	245.71	14.55	204.282	203.05
2	599.28	240.79	14.75	212.544	213.12
3	687.66	243.45	14.37	220.994	219.236
4	680.44	244.41	14.43	233.1	233.753

5. Acknowledgements

6. Conflict of Interest

The authors report no conflict of interests relevant to the subject of the present manuscript.

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ممانعت از تشکیل آکریلامید هنگام پخت نان ورآمده با مخمر با افزودن آسپاراژیناز، زمان و دمای پخت، به روش سطح پاسخ

مشاعر ماتوری، ایران عالم زاده*

گروه مهندسی شیمی و نفت، دانشگاه صنعتی شریف، تهران، ایران.

چکیده

سابقه و هدف: آکریلامید ترکیبی سمی برای انسان است که در اثر واکنش میلارد در درجه حرارت بالا تولید می‌شود. در این تحقیق این واکنش می تواند با استفاده از آنزیم آسپاراژیناز، کنترل زمان و درجه حرارت پخت نان ورآمده با مخمر مهار شود.

مواد و روش‌ها: در این مطالعه؛ برای مطالعه اثر آسپاراژیناز (۹۰۰-۳۳۰ واحد در کیلوگرم آرد)، درجه حـرارت پخت (۲۸۰-۲۳۰°C) و زمان پخت (۱۶-۱۳ دقیقه) بر تشکیل آکریلامید در نان گندم ورآمـده بـا مخمـر از روش سطح پاسخ، ۵ –سطح-۳ عاملی طراحی مرکب مرکزی، استفاده شد.

یافته‌ها و نتیجه‌گیری: افزودن آسپارازیناز اثر کاهشی بر تشکیل آکریل آمید را نشان داد ($p \leq 0/0001$). درجه حرارت پخت به طور قابل معنی داری میزان آکریل آمید در نان را افزایش داد ($p \leq 0/0001$). همبستگی قوی بین درجه حرارت پخت و تشکیل آکریل آمید مشاهده شد. مدت زمان پخت و اثر متقابل آن بر آسپارازیناز کم ولی بر کاهش میزان آکریلامید در نان معنی دار بود ($p \leq 0/0001$). سه عامل درجه حرارت پخت، مدت‌زمان پخت و غلظت آنزیم با استفاده از روش سطح پاسخ بهینه شد و مقادیر به‌دست آمده به ترتیب ۲۵۴/۷۱°C، ۱۴/۵۵ دقیقه و ۷۵۲/۱۵ U Kg^{-1} بود. فرایند آنزیمی به عنوان روشی سالم و مناسب برای پیشگیری از تشکیل آکریلامید در نان می تواند پیشنهاد شود.

تعارض منافع: نویسندگان اعلام می کنند که هیچ تعارض منافعی وجود ندارد.