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Inhibitory effect of acylated anthocyanins on heterocyclic amines in grilled chicken breast patty and its mechanism

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A R T I C L E I N F O

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A B S T R A C T

Heterocyclic amines (HCAs) are a group of carcinogenic substances produced in protein-rich poultry meat under high-temperature. Enzymatic acylation of anthocyanins (ACNs) is a reliable way to improve their stability, and we recently found the acylated cyaniding-3-O-glucose (cyanidin-3-6-cinnamoyl-glucoside, C3(6C)G) could effective inhibit the HCAs formation, but the underline mechanism was still obscure. Thus, the present study investigated the inhibitory effect ofC3(6C)G on HCAs formation in the food system (chicken breast) and to explore the potential mechanism. The results showed that C3(6C)G with different concentrations (0.1, 0.5 and 1.0 mg/mL) could significantly inhibit lipid oxidation and decrease the total HCAs content ($P<0.05$) in chicken breast meat patty after roasting. The samples with 0.1 mg/mL C3(6C)G had the best inhibition effect on total HCAs, with an inhibition rate of 28%, and the inhibition rates for IQ, Harman, TRP-P-2, PhIP and AαC were 34%, 46%, 100%, 54% and 41%, respectively.

1. Introduction

Chicken breast contains substantially high amounts of protein, vitamins and other nutrients, and is low in calories and fat, it has become the best choice for more and more people who pay attention to fitness and health. Heterocyclic amines (HCAs) are mutagenic/carcinogenic heterocyclic aromatic compounds produced by high-temperature processing of protein-rich foods. They are formed by the Maillard reaction or the pyrolysis of proteins, which can induce a variety of cancers, causing great harm to the human body (Pleva et al., 2020). So far, more than 30 HCAs have been detected in food and chemical model systems. Studies have shown that the formation of HCAs in meat products could be affected by many factors, among which antioxidant is one of the important factors affecting the formation of HCAs (Ahn & Grun, 2005).

The chemical structure of ACNs is believed to be a major factor influencing the stability of these pigments. ACNs may be glycosylated and acylated by different sugars and acids. Acylation of the ACNs molecule is reported to improve stability to these pigments(Jokioja et al., 2021).Enzymatic modification is a reliable method for the acylation of ACNS, and single acylated anthocyanin is able to be obtained after isolation and purification processes. Acylated ACNs are active substances with a great application potential, but most studies involved are bioactivity evaluation at molecular and cellular levels, and the application in practical scenarios is still absent (Teng et al., 2022).

Therefore, this study supplemented the acylated ACNs into chicken breast patty, and to investigated the inhibitory effect of different

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concentrations (0 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.0 mg/mL) of C3 (6C)G on the formation of HCAs in grilled chicken breast patty. The underline mechanism was tried to explain by inspecting the precursor substances (creatine, creatinine, glucose and TBARs content) and their relationship. It provides a basis for finding high quality food additives to reduce the harm of HCAs, so as to improve food safety and quality.

2. Materials and methods

2.1. Materials and reagents

Fresh chicken breast was purchased from a local market (Zhanjiang, Guangdong province, China), cleaned, removed the fascia, and minced using a grinder. Cyanidin-3-O-glucose (C3G) with a purity of 98% was ordered from Durst Biotechnology Co., LTD (Chengdu, China). HCA standards of IQ、MeIQ、MeIQx、4,8-DiMeIQx、Norharman、Harman、PhIP、AαC、MeAαC、Trp-P-2、Trp-P-1A were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Chromatographic grade of methanol, acetonitrile, ammonium acetate, triethylamine, phosphoric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate and other analytical grade reagents were bought from Sinopharm chemical reagent Co., LTD (Beijing, China). Methyl cinnamate and 4A molecular sieve of analytical grade were ordered from Maclin Biochemical Technology Co., LTD (Shanghai, China). Novozym 435 lipase was purchased from Novozymes Biotechnology Co., LTD (Shenyang, China).

2.2. ACN acylation and purification

C3G, methyl cinnamate, Novozym435 lipase, and steam flask were dried in a vacuum dryer at 35 °C for 24 h (Chandra et al., 2021; Zhu et al., 2021) Pyridine was placed in an activated 4A molecular sieve for 48 h for later use. C3G of 20 mg was weighed and put into a distillation flask, then added with 5 mL pyridine, and the flask was placed in an ultrasonic instrument for 2 min to completely dissolve C3G. After that, methyl cinnamate of 1 g and Novozym 435 lipase of 200 mg was added, and the flask was fitted to a rotary evaporator to avoid the light reaction. The acylation was proceeded at 40 °C and −0.91 Kpa for 48 h with the rotation speed at 45 r/min.

Semi-preparation liquid phase purification was employed for the purification of acylated ACNs. The pre-treatment was as follows: the acylated mixture was transferred to a 50 mL centrifuge tube, and five times the volume of petroleum ether was added for extraction of water at 40 °C for 10 min. The tube was then centrifuged at 3000 r/min for 10 min, and the supernatant was poured to remove most of the impurities such as methyl cinnamate. After the above extraction and centrifugation steps were repeated twice, an appropriate amount of methanol was added into the centrifuge tube to release C3G and acylated C3G from the enzyme, and the Novozym435 enzyme in the solution was removed by passing through 0.22 μm filter membrane. Finally, the solution was dehydrated by a constant blow of nitrogen. The semi-prepared liquid chromatography was performed on A BDS HYPERSIL C18 (250 mm × 10 mm I.D., 5 μm) column, and mobile phase A was composed of formic acid aqueous solution (0.1%, V/V) and mobile phase B was composed of acetonitrile. The elution gradient was 0–20 min, 5–22% B. 20–40 min, 22–60% B; 40–60 min, 60-5% B; 60–65 min, 5% B. The flow rate was 3.0 mL/min, column temperature was set at 30 °C And the injection volume was 50 μL. The detection wavelength was 520 nm and 280 nm.

2.3. The preparation of chicken breast patty

The acylated ACN was dissolved in diethylene glycol, and 1 mL of C3 (6C)G sample solution at concentrations of 0, 0.1, 0.5 and 1.0 mg/mL was added to 40 g chicken breast meat with fascia removed, and the meat was evenly pressed on a 6 cm petri dish. The chicken breast patty was grilled in an oven at 230 °C for 20 min, with 10 min intervals to turn it over. After being taken out, the samples were cooled naturally at room temperature, weighed, and then homogenized into powder with a crusher, and stored in a refrigerator at −80 °C. In addition, no salt, flavor, fat or oil was added to the sample.

2.4. Cooking loss, lipid, protein, moisture, pH determination

PH value, moisture, lipid and protein content of samples were determined according to standard food protocols (Bohrer, 2017). The atmospheric drying method was employed to determine moisture content, and lipid content was determined by the Soxhlet extraction Chicken breast samples were weighed before and after baking to calculate the cooking loss.

$$\text{Cooking loss (\%)} = (M1\text{-}M2)/M1) \times 100$$

Where, M1 was the quality of chicken breast sample before baking.

M2 was the quality of chicken breast sample after roasting.

2.5. Solid phase extraction (SPE) for HCAs

The dried and crushed chicken breast sample (5 g) was accurately weighed and transferred into a 50 mL centrifuge tube, and 30 mL 1 M NaOH solution was added. The samples were dispersed evenly for 1 min using a hand-held homogenizer, and then transferred to a 250 mL beaker, in which 15 g diatomite was added. After mixing evenly, 40 mL ethyl acetate was added. The beaker was placed in a cleaning ultrasonic instrument for 30 min, and the supernatant was transferred to the extraction device. Then 40 mL ethyl acetate was added to the beaker. After the supernatant was combined, the beaker was re-extracted and the upper layer was removed from the solution.

The SPE for HCAs was employed as follows (Khan et al., 2015): 5 mL dichloromethane was naturally passed through the PRS column (500 mg/3 mL) to activate the column, and then the sample extract was naturally and slowly passed through PRS column to adsorb the sample onto the column. The prepared 6 mL 0.1 M hydrochloric acid, 15 mL methanol-0.1m hydrochloric acid (5/5, v/v) and 2 mL water were successively drained through the PRS column and collected into 50 mL centrifuge tube to obtain the non-polar HCAs part, which was neutralized by adding 1 mL concentrated ammonia water. The C18 column was activated with 5 mL water and 10 mL methanol, and the non-polar HCAs part of the centrifugal tube was naturally and slowly passed through the C18 column. The impurities were removed by rinsing with 2 mL of water, and the water was drained. Then 2 mL eluent (methanol: 25% ammonia (9:1, V/V) was passed through a C18 column and drained to elute non-polar heterocyclic amines from the C18 column and the eluent was collected into a 10 mL centrifuge tube. Ammonium acetate (pH = 8.0, 0.5 M) of 20 mL was used to elute the polar HCAs in PRS onto the C18 column through the connected PRS-C18 column. Finally, the C18 column was eluted with 2 mL eluent (methanol: 25% ammonia water (9:1, V/V)), and the eluent was collected into the centrifugal tube to combine the polar HAAs and non-polar HCAs. The sample nitrogen in the centrifuge tube was blown dry, dissolved in 0.3 mL methanol, and filtered by 0.22 μm microporous membrane for further analysis.

2.6. HPLC - DAD/FLD quantification for HCAs

HCAs analysis was performed using a high-performance liquid chromatography ultraviolet detector combined with a fluorescence detector (Scott et al., 2007). The column of TSK-Gel ODS-80 TM (5 μm, 25 mm × 4.6 mm.80 A; Tosoh, Tokyo, Japan) was used for the separation and the column temperature was set at 30 °C. The mobile phase consisted of solvent A: 0.01 M triethylamine (phosphoric acid adjusted to pH 3.2), solvent B: acetonitrile, and solvent C: 0.01 M triethylamine (phosphoric acid adjusted to pH 3.6). Elution gradient was set as follows: 0–10 min, 5%–15% B; 10–20 min, 15%–25% B; 20–30 min, 25%–55% B;

30–35 min, 55%–80% B, flow rate was 1 mL/min. The injection volume was 10 μL.

The UV detection wavelength for IQ, MeIQ, MeIQx, and 4, 8-DimeiqX was 263 nm. The fluorescence detection wavelength for harman, AαC and MeAαC was 265/440 nm. The fluorescence detection wavelength for Norharman, PhIP, TRP-P-1 and TRP-P-2 was 265/410 nm.

2.7. Determination of thiobarbituric acid reactants (TBARS)

TBARs content was determined according to the method of de Sousa et al. (2020) with some modifications. Firstly, solution A (TBA solution) was prepared as follows: 0.2 g of 2-thiobarbituric acid was replaced in a volumetric flask and adjusted the volume to 100 mL with 1-butanol. Solution B (TCA-HCL solution) was prepared by dissolving 16.8 g trichloroacetic acid with a small amount of 0.125 M HCl solution, transferred into a volumetric flask, and diluted into 100 mL with 0.125 M HCl solution. The crushed chicken breast sample (0.3 g) was mixed with 3 mL solution A and 17 mL solution B, placed in a water bath and reacted at 100 °C for 30 min. After that, the sample was refrigerated, and 5 mL chloroform was added, evenly swirled, and centrifuged at 3000 rpm for 10 min. Finally, the supernatant was collected and three groups of each sample for a parallel experiment. The absorption wavelength was measured at 532 nm using a microplate reader.

TBARS (mg/kg) = (A_532 / W) x 9.48

Where, absorption value at A532:532 nm;
W: The quality of sample.

2.8. Determination of glucose content

The glucose content was determined according to Serpen and Gökmen (2009) with a slight modification. Preparation of DNS reagent: 315 mg of 3, 5-dinitrosalicylic acid was precisely weighed and transferred into a beaker, added with 50 mL of water, and dissolved in a water bath at 45 °C. Sodium hydroxide (NaOH) of 2 g was added into the beaker carefully with a small amount each time. After dissolution, 9.1 g of sodium potassium tartrate tetrahydrate, 250 mg of phenol, 250 mg of anhydrous sodium sulfite, and 30 mL of water were mixed respectively. Finally, the mixture was transferred into a brown volumetric flask and diluted to 100 mL, and then the DNS solution was stored at room temperature for a week before use.

The treatment of roast chicken breast samples proceeded as follow, 3 mL glacial acetic acid and 21.9 g zinc acetate were placed in a volumetric flask and diluted to 100 mL with distilled water (solution A); potassium ferrocyanide of 10.6 g was transferred into a volumetric flask and diluted to 100 mL with distilled water (solution B). Four gram of chicken breast sample was weighed and put into a centrifuge tube, adding with 5 mL of water, 1.5 mL of liquid A, and 1.5 mL of liquid B, respectively. After 15 min reaction in a water bath at 50 °C, the mixture was centrifuged at 4000 rpm for 7 min, and the supernatant was collected in 25 mL volumetric flask. The centrifugation process was repeated three times, the supernatant was combined and the final volume was fixed to 25 mL for analysis.

2.9. Determination of creatine and creatinine

Creatine and creatinine contents in the chicken breast sample were determined according to previous reports ((Dunnett et al., 1991; Elbir & Oz, 2020). Homogenized and dried roasted chicken breast sample of 200 mg was accurately weighed and transferred into a 10 mL centrifuge tube, and 2 ml of precooled 0.5 mol/L perchloric acid solution was added, and an ultrasound was performed in an ice water bath for 10 min. The supernatant was centrifuged at 4 °C at a speed of 10000 r/min for 10 min. The same amount of 5 M KOH solution was used to adjust the pH to 6.5–7. The supernatant was centrifuged again under the above conditions, and the supernatant was combined to adjust the final volume of 10 mL and filtered by 0.22 μm filter membrane.

HPLC-DAD was employed for the detection and conditions were as follows: An InertSustain C18 (150 mm × 4.6 mm I.D., 5 μm) column was used. Mobile phase A consisted of 10 mmol/L sodium dihydrogen phosphate and the buffer solution of disodium hydrogen phosphate (phosphoric acid adjusted to pH 6.2), and mobile phase B consisted of methanol. The elution gradient was as follows, and the column temperature was 30 °C. The injection volume was 10 μL. The detection wavelength was 210 nm. The elution gradient was 0–5 min 100% A, and the flow rate was 0.5 mL/min 5–7 min 100% A, flow rate 0.7 mL/min; 7–22 min 30%A, 70%B, flow rate 0.7 mL/min; 22–23 min 100%a, flow rate 0.5 ml/min; 23–30 min 100%A flow rate of 0.5 mL/min.

2.10. The data analysis

The experiment was repeated in three sets, and the results were expressed as mean ± standard deviation. SPSS22.0 was used for significance analysis of intra- and intergroup data. SPSS 25.0.0 software was used for one-way ANOVA and origin 2018 software was used for plotting. $P < 0.05$ indicates significant difference. .

3. Results and discussion

3.1. Identification of C3(6C)G

HPLC analysis results showed that the conversion rate for enzymatic acylation of C3G reached 80%, and the purification process using semi-preparative HPLC increased the purity of C3(6C)G to 98.9%. In order to confirm the specific structure of the C3(6C)G, coupled tandem mass spectrometry was employed. Fig. 1A shows that a molecular ion peak [M+H] with a signal peak of 579 appeared, which was the sum of the molecular weight of one cinnamate acyl group (147) and one C3G (449) without hydroxyl group, indicating that the acylation reaction produces a monoacylated C3G. In the secondary mass spectrum (Fig. 1B), the emergence of a molecular weight of 287 ion fragments, which is obtained by removing a glycosidase from C3G, and the fragments for the molecular weight of 287 gradually increased with the enhancement of collision induced dissociation (CID) as induced by the secondary mass spectrometry. But there were no other ion fragments appeared, which showed that the mother nuclear stability of C3G was strong. Besides, the acylation occurred on glucoside, which was consistent with previous publications(Liu et al., 2020; Teng et al., 2020), and the acylation reaction site catalyzed by Novozym 435 was specific. The specific reaction equation for the C3(6C)G is shown in Fig. 1C, and the monoacylation product of C3(6C)Gwas obtained.

3.2. The effect of C3(6C)G on cooking loss, lipid, protein, moisture, pH value of grilled chicken breast patty

Fig. 2A shows the chicken breast meat prepared by adding different concentrations of C3(6C)G into the raw chicken breast meat. The meat patties with 0, 0.1, 0.5 and 1.0 mg/mL C3(6C)G were marked as blank, low, medium, and high dose groups, respectively. The meat patties have good color, uniform shape and no obvious bubbles on the surface, which was suitable for analysis.

The cooking loss, moisture, pH, protein and lipid contents of chicken breast samples are shown in Table 1. In the samples without C3(6C) Gtreatment, cooking loss, moisture, pH, protein and lipid contents were 40.09, 0.58, 6.23, 38.46 and 0.032, respectively, which were mostly consistent with the results of a previous study (Tengilimoglu-Metin & Kizil, 2017). Changes in the physical state and content of water affect the secondary or tertiary structure of proteins and may expose specific binding sites of proteins to free HCAs, thereby promoting the production of protein-bound HCAs. It could be noted that with the increase of concentration of C3(6C)G added to chicken breast meat, cooking loss

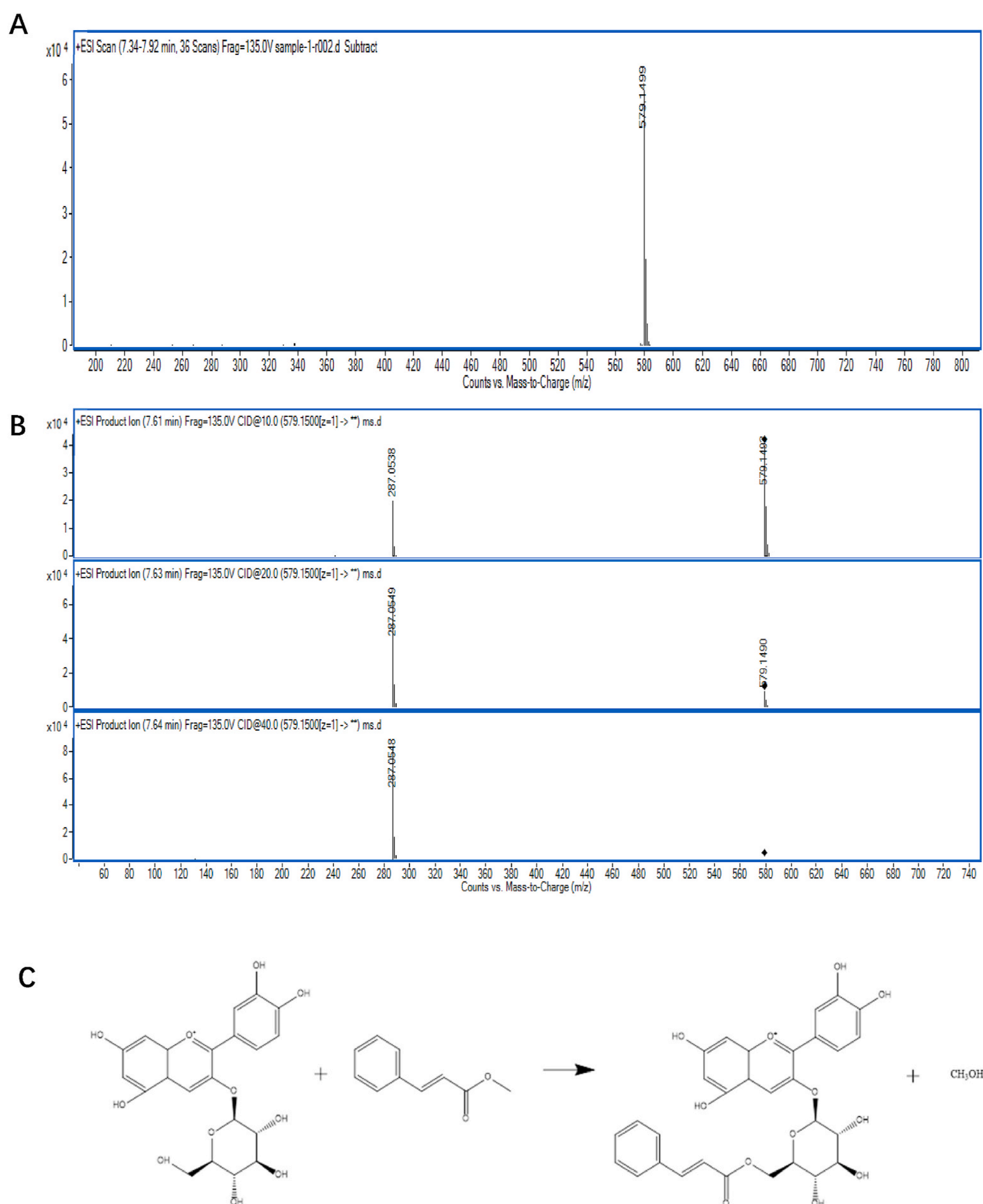

Fig. 1. Acylation pathway of C3G with methyl cinnamate (A) and the primary (B) and the secondary (C) mass spectrometry of acylated anthocyanin.

decreased substantially, water content increased significantly, but no obvious change in pH was observed. It suggested that the supplementary of C3(6C)G could effectively reduce the water loss of chicken breast in the cooking process, and higher concentration of C3(6C)G was benefit for higher moisture retention rate of the sample. Moreover, protein and lipid contents in chicken breast are important indicators to measure its product quality. Table 1 revealed that no significant difference was checked for the lipid content of grilled chicken breast after adding with various concentration of C3(6C)G, but high (1.0 mg/mL) and medium (0.5 mg/mL) supplements led to a significant decrease in protein content, which might be associated with the increased moisture content.

3.3. Inhibitory effect of C3(6C)G on HCAs formation in grilled chicken breast patty

Fig. 2 shows the UV (B) and fluorescence (C) spectra of HPLC for 11 kinds of mixed HCAs standard solutions. The peak times were IQ, 9.8 min; MeIQ, 11.2 min; MeIQx, 13.1 min; 4, 8-dimeiqx, 16.4 min; Norharman, 16.9 min; Harman, 18.9 min; Trp-p-2, 21.7 min; PhIP, 22.5 min; Trp-p-1, 23.8 min; AαC, 28.0 min; and MeAαC, 30.2 min. Table 2 shows the effects of different supplement concentrations of C3(6C)G on HCAs content in grilled chicken breast samples. The total HCAs amount detected in the blank group was 6.89 ng/g, including IQ and MeIQ. The contents for MeIQx, 4, 8-Dimeiqx, PhIP, Norharman, Harman, Trp-p-2, TRP-P-1, and AαC were 0.1, 0.23, 0.30, 2.19, 0.40, 1.77, 0.55, 0.67, 0.25 and 1.42 ng/g, respectively. MeAαC was not detected in either of samples, and 4, 8-Dimeiqx was found to be the most abundant HCAs in breast patties.

Fig. 3(A) and (B) show the PLS-DA score and load diagram of HCAs in the sample of grilled chicken breast added with different concentrations of C3(6C)G. The sum of PC1 and PC2 is 90.5% (>50%), which represents 90.5% of the results can be explained by the analysis diagram. The HCAs content data of the blank group and C3(6C)G group are suitable for partial least square discriminant analysis with high reliability and validity. As can be seen, only the blank group was distributed in the second quadrant, and the positions of C3(6C)G were different, indicating that the HCAs content in the blank group was significantly different from that in the C3(6C)G supplemented groups ($P<0.05$). In addition, the high-

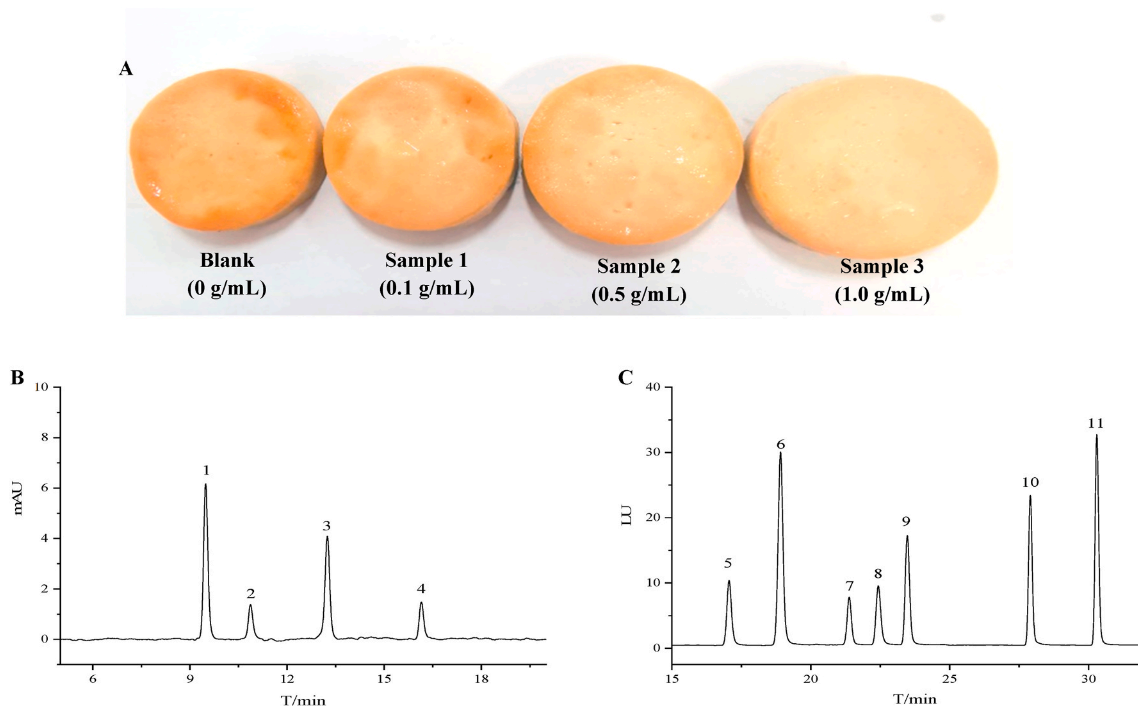

Fig. 2. (A) The effect of different concentrations of acylated anthocyanin (C3(6C)G) on the appearance of baked chicken breast patties, and HPLC chromatograms of 11 HAAs standards by (B) UV detection and (C) fluorescence detection. Peak information: 1.IQ; 2.MeIQ; 3.MeIQx; 4.4,8-DiMeIQx; 5.Norharman; 6.Harman; 7.Trp-P-2; 8.PhIP; 9.Trp-P-1; 10.AαC; 11.MeAαC.

Table 1
Effects of different concentrations of C3(6C)G on cooking loss, moisture, pH, protein and lipid content of roasted chicken breast patties.

Supplemented Conc. of C3(6C)G (mg/mL)	Cooking loss(%)	Moisture (g/g)	pH	Protein content (g/100g)	Lipid content (g/g)
0	40.09 ± 0.37a	0.58 ± 0.001d	6.23 ± 0.02a	38.46 ± 0.44a	0.032 ± 0.003a
0.1	38.29 ± 0.76 ab	0.59 ± 0.007c	6.20 ± 0.03a	38.04 ± 0.15a	0.031 ± 0.002a
0.5	39.58 ± 0.95 ab	0.60 ± 0.001b	6.19 ± 0.05a	37.19 ± 0.39b	0.029 ± 0.002a
1	37.66 ± 0.48b	0.61 ± 0.003a	6.17 ± 0.03a	36.81 ± 0.29b	0.029 ± 0.002a

dose group was mainly distributed in the first quadrant, the medium-dose group was mainly distributed in the third quadrant, and the low-dose group was all in the fourth quadrant, indicating that the sensitivity of HCAs was variable when different concentrations of C3(6C)G were supplemented into chicken breast meat. It can also be seen from the score chart that the distance between the low-dose group and the high-dose group was further than that of the blank group, suggesting that the similarity between the low-dose and high-dose groups was lower than that of the blank group. Similar results were obtained in Table 2. The sample with 0.1 mg/mL C3(6C)G had the best inhibition effect on total HCAs in grilled chicken breast meat. The position of blank groups in the score plot is similar to PhIP, harman, norharman, 4, 8-dimeiqx, and TRP-P-1 in the loading plot, suggesting that C3(6C)G could effectively inhibit these HCAs.

C3(6C)G could significantly inhibit the above five HCAs species ($P<0.05$), and the inhibition rates of harman, norharman, 4, 8-Dimeiqx and TRP-P-1 were dependent on its concentration. Within the selected concentration range, the higher concentration of C3(6C)G, the better effect was found. Although low concentration of C3(6C)G had no

Table 2
Effects of different concentrations of C3(6C)G on HCAs content of roasted chicken breast samples.

A cylated ACNs con (mg/g)	IQ	MEIQ	MeIQx	4,8-DiMeIQx	Norharman	Harman	Trp-P-2	PhIP	Trp-P-1	AαC	MeAαC	Total HAAs content
	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)
0	0.10 ± 0.02b	0.23 ± 0.07c	0.27 ± 0.03a	2.19 ± 0.12a	1.77 ± 0.04a	0.55 ± 0.02a	0.67 ± 0.05a	0.40 ± 0.014a	0.25 ± 0.01a	1.42 ± 0.12c	Nd	6.89 ± 0.39a
0.1	0.07 ± 0.00c −34%	0.25 ± 0.01a (-8%)	0.28 ± 0.07a (-3%)	1.38 ± 0.03b −37%	1.49 ± 0.04b −16	0.30 ± 0.004b −46%	Nd −100%	0.18 ± 0.02b −54%	0.18 ± 0.02b −30%	0.84 ± 0.09d −41%	Nd	4.96 ± 0.15d −28%
0.5	0.11 ± 0.01a (-14%)	0.24 ± 0.03b (-4%)	0.20 ± 0.01b −25%	1.11 ± 0.07b −50%	1.43 ± 0.07b −19%	0.30 ± 0.003b −46%	0.37 ± 0.08a −46%	0.20 ± 0.016bc −50%	0.15 ± 0.01c −42%	1.63 ± 0.10b (-15%)	Nd	5.73 ± 0.2b −17%
1	0.10 ± 0.00b −2%	0.23 ± 0.01c (-1%)	0.07 ± 0.001c −72%	Nd 100%	1.24 ± 0.08c −30.00%	0.27 ± 0.002b −51%	0.65 ± 0.04a −3%	0.21 ± 0.013b −47%	Nd −100%	2.40 ± 0.14a (-69%)	Nd	5.18 ± 0.08c −25%

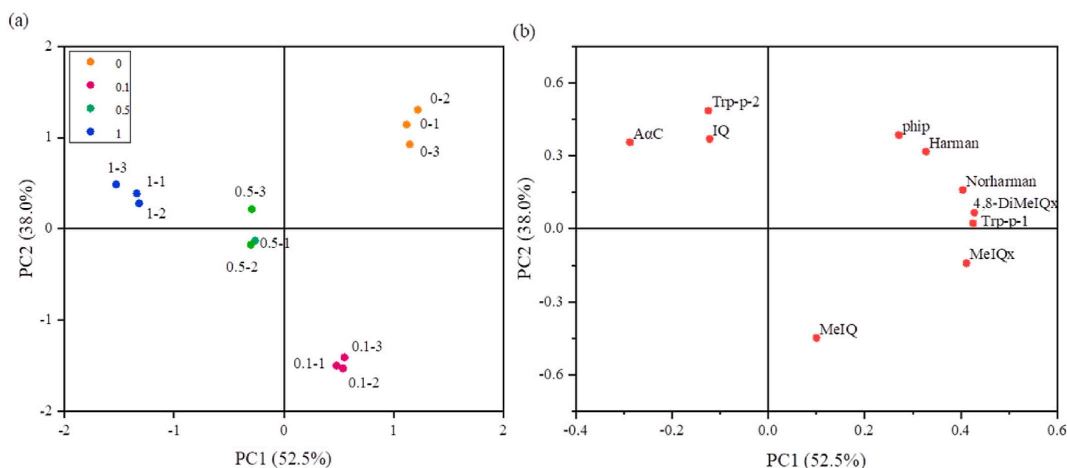

Fig. 3. (a) Scores plot and (b) loadings plot of principle component analysis (PCA) of HCAs in roasted chicken breast patties supplemented with different concentrations of acylated anthocyanin (C3(6C)G).

significant effect on MeIQx, the content of MeIQx decreased significantly with the concentration increase, and the inhibitory rate was as high as 72%. In contrast, the inhibitory rate of TRP-P-2 decreased with the increase of C3(6C)G concentration. Trp-p-2 had the best inhibition effect in low-dose roasted chicken breast samples, with the inhibition rate reaching 100%. The contents of IQ, Phip and AαC were also the lowest in the low-dose group, while in the medium-high dose group, the inhibitory effect was low. The addition of C3(6C)G had a promoting effect on MeIQ production in roast chicken breast meat, but with the increase in dose, the promoting effect decreased, and there was no significant difference between MeIQ content and blank group when the dose concentration increased to 1.0 mg/mL (P>0.05).

In conclusion, although different additive concentrations of acylated anthocyanin (C3(6C)G) have different effects on HCAs in broiled chicken breast samples, C3(6C)G can still be added to chicken breast as a food additive to inhibit the generation of total HCAs. Among the three selected supplemental levels, only a small amount was required to achieve the best effect on the overall inhibition against HCAs, and it is noteworthy that the inhibition of IRAC published class 2A carcinogen (IQ) was also very good at the low supplemental level.

3.4. Inhibitory effect of C3(6C)G on HCA's precursor and TBARS in grilled chicken breast patty

Under the heating process of meat products, creatine can be converted into creatinine, and the creatinine is the necessary precursor of IQ and PhIP, which has a certain promoting effect on HCAs formation (Gibis & Loeffler, 2019). On the other hand, glucose is considered as

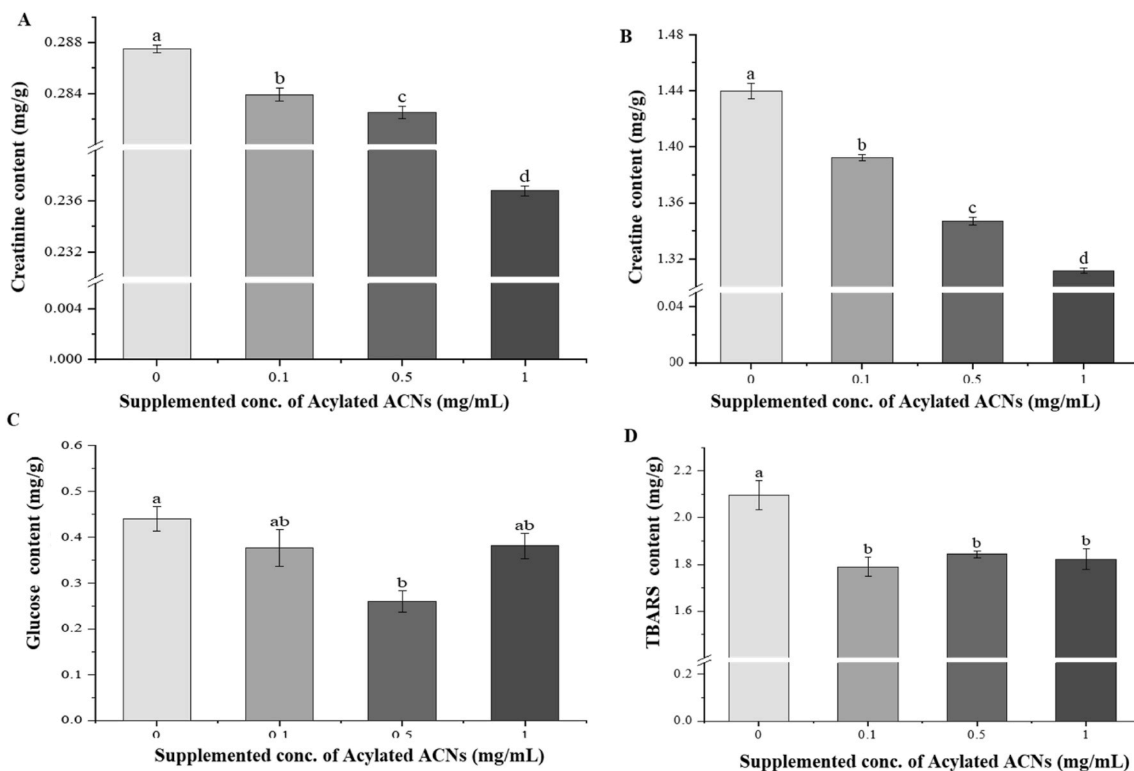

Fig. 4. Effects of different concentrations of acylated anthocyanin (C3(6C)G) on (A) creatinine content, (B) creatine content, (C) glucose content, and (D) TBARs content in roasted chicken breast samples ($P < 0.05$).

another important precursor of many HCAs, and the concentration of precursor is positively correlated with their contents. Therefore, the contents of creatine, creatinine, and glucose in the grilled chicken breast after the supplementation of various concentrations of C3(6C)G were analyzed and tried to find out the underlying mechanism of inhibition.

Fig. 4 (A) and 4 (B) show the contents of creatinine and creatinine in roast chicken breast samples with different supplemental concentrations of C3(6C)G. The highest creatinine (0.28 mg/g) and creatine (1.44 mg/g) contents were noted in the samples without C3(6C)G. In addition, the creatine and creatinine contents decreased significantly with the increased concentration of C3(6C)G ($P<0.05$), indicating the C3(6C)G was effective in the inhibition of creatinine and creatine generation. Table 2 also displayed that the concentration of C3(6C)G was also another influencing factor for HCAs' formation, suggesting that the C3 (6C)G might inhibit the formation of HCAs through reducing the contents of precursor (creatinine and creatine) to reduce aldol condensation reaction with aldehydes, pyridine or pyrazine.

Besides, the positive correlation effect of glucose content on PhIP depends on its available concentration. A low concentration of glucose could promote PhIP formation, while a high concentration of glucose (molar ratio of total creatine to glucose >0.5) may reduce PhIP formation. Gibis and Loeffler (2019) found that the addition artichoke extract to grilled chicken breast patty correlated with the content of PhIP and the creatine/glucose molar ratio. Fig. 4 (C) shows the effects of different addition concentrations of C3(6C)G on glucose content in grilled chicken breast meat samples. It could be observed that the glucose content in the blank group was 0.44 mg/g, and other groups with the supplement of various C3(6C)G concentrations showed a slight descent in glucose content, indicating the glucose degradation effect of C3(6C)G during the cooking process of chicken breast patty. Ahn and Grun (2005) also observed that glucose content in beef was reduced by the addition of several natural extracts. On the other hand, the corresponding relationship between PhIP and creatine/glucose was not found in this study, which may be because C3(6C)G not only affect glucose content, but also significantly affect creatine/anhydride content.

In many related studies (Xu et al., 2017; Zamora et al., 2020), lipid oxidation may generate a large number of free radicals and intermediates that can attack amino acids to produce active carbonyl products (acetaldehyde and α -ketoic acid). These key products of the Maillard reaction, which enable to promote HCAs formation. Jing et al. (2022) found that oxidation of tilapia increased the formation of PhIP in grilled fish slices, and the lipid peroxidation product acrolein had the comprehensive ability to promote Strecker degradation of phenylalanine and react with phenylalanine, creatinine and PhIP, further supporting the potential contribution of lipid oxidation products to PhIP formation. Xue et al. (2022) found that ginger and curcumin inhibited the production of free and bound HCAs in roast beef patty by alleviating lipid peroxidation, quenching alkyl free radicals and inhibiting active carbon-based intermediates. Wu et al. (2021) also studied the influence of freeze-thaw cycles on lipid oxidation and its relationship with the formation of HCAs in raw meat, and found that norharman and harman showed a significant positive correlation with thiobarbituric acid reaction substances.

Fig. 4 (D) displays the TBARS value of chicken breast samples ($P<0.05$) was significantly reduced when adding with different concentrations of C3(6C)G. These results indicated that the free radical scavenging ability of C3(6C)G as an antioxidant could significantly alleviate the lipid peroxidation of chicken breast during high-temperature cooking, thus achieving HCAs inhibitory effect.

3.5. *Potential inhibitory mechanism of C3(6C)G on HCAs in grilled chicken breast patty*

In order to further study the relationship between HCAs formation and precursor substances, the spearman algorithm was used to study the correlation between HCAs content and related physical and chemical indexes (Fig. 5). The figure shows the positive and negative relationship between different kinds of HCAs in each group and related physical and chemical indexes. Red represents positive correlation, blue represents negative correlation, and the depth of color indicates the strength of correlation.

The moisture content in chicken breast patty was negatively correlated with most HCAs, which was consistent with the previous conclusion that the addition of water-retaining substances would reduce HCAs content. Degradation of creatine and creatinine was significantly associated with the most of HCAs, including MeIQx, 4.8-DImeiqX, Norharman, Harman, and TRP-P-1. The correspondence between creatine and its precursor has long been verified, which is consistent with the correlation results obtained in this study. It further indicates that creatine and creatinine are important precursor compounds for the formation of heterocyclic amines. Glucose, another important precursor of HCAs, was closely correlated with PhIP, TRP-P-1, 4.8-dimeiqx, and norharman in this study. The corresponding relationship between the degree of lipid peroxidation in the cooking process of meat products and HCAs generated in the final system has long been demonstrated. We also found that TBARS values were correlated with the most of HCAs. It is worth noting that the content formation rule of AαC seems to be contrary to that of other heterocyclic amines. This may be because in the generated HCAs, AαC, is formed by high-temperature pyrolysis of proteins or amino acids, and the heat transfer efficiency of the system increases due to the loss of water, while norharman and harman, both HCAss, are not affected due to their ability to form at lower temperatures.

Hence, C3(6C)G may inhibit the formation of HCAs in grilled chicken breast by maintaining moisture in chicken breast, inhibiting glucose and creatine/anhydride of HAAs precursor, and alleviating lipid peroxidation during chicken breast cooking.

4. Conclusion

In this experiment, C3(6C)G was synthesized using enzymatic modification. The inhibition of C3(6C)G on HCAs formation in grilled chicken breast meat (230 °C, 20 min) was studied. The results showed that the total amount of HCAs was significantly decreased. It was found that C3(6C)G had a dose-dependent effect on the water retention of grilled chicken breast patty, but little effect on other physicochemical properties (protein, lipid content and pH value) was confirmed. In the group without C3(6C)G, the total HCAs production was 6.89 ng/g, and different supplementary concentrations of C3(6C)G showed a significant

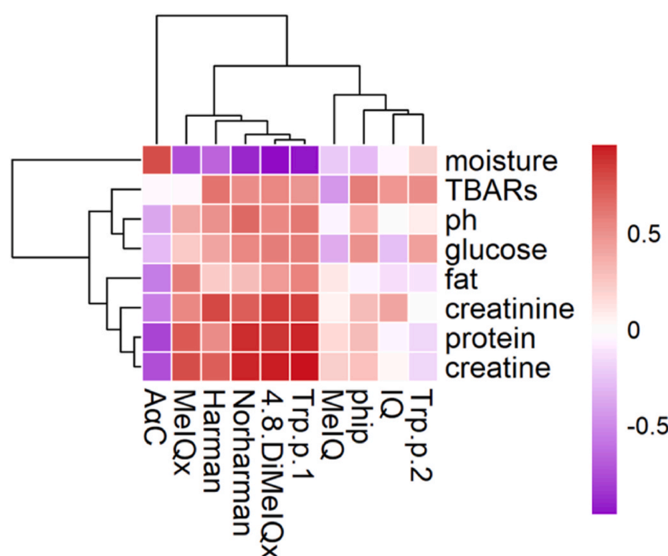

Fig. 5. Heat map of associations between HCAs and their precursors in grilled chicken breast patties supplemented with acylated anthocyanin (C3(6C)G).

inhibition effect on total HCAs production. Besides, the addition of C3(6C)G could significantly inhibit the glucose and creatinine contents, slowing down the lipid peroxidation in the process of chicken breast cooking. According to the correlation analysis of various physical indexes and HCAs, the possible inhibition mechanism of the C3(6C)G on HCAs in chicken breast patty might via maintaining the moisture content in chicken breast to inhibit the generation of related HCAs precursor (glucose and creatine/anhydride), alleviating lipid peroxidation during high-temperature cooking. Therefore, C3(6C)G can be used as functional food additives in the production of chicken breast meat to inhibit the generation of HCAs.

CRediT authorship contribution statement

Hui Teng: Methodology, Writing – original draft, Writing – review & editing. **Yani Mi:** application of statistical, mathematical, computational. **Hongting Deng:** application of statistical, mathematical, computational, Formal analysis. **Yuanju He:** preparation, creation and/or presentation of the published work. **Shunxin Wang:** preparation, creation and/or presentation of the published work. **Chao Ai:** preparation, creation and/or presentation of the published work. **Hui Cao:** Management and coordination responsibility for the research activity planning and execution. **Baodong Zheng:** Management and coordination responsibility for the research activity planning and execution. **Lei Chen:** Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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