Alternation of aroma and flavor profiles in biscuits by an enzymatic approach

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Abstract. Bromelain is a complex protease existing in both the fruits and the stems of pineapple plants. Bromelain from these two parts of pineapple plants exhibits different characteristics. Stem bromelain has been extensively investigated and is commercially available for applications in various industries. On contrast, studies on fruit bromelain are quite limited. Here we investigated the effects of fruit bromelain on production of biscuits aroma and flavor compounds. The fruit bromelain was first isolated from pineapple fruit, activity assayed, purity of enzyme determined and then added into dough. Biscuits aroma and flavor compounds were identified by HS-SPME combined with GC-MS. Results showed that aroma and flavor compounds of biscuit varied in terms of amount and varieties.

1. Introduction
Pineapple is a widely grown fruit in tropical and subtropical regions and is a natural source for bromelain [1]. Bromelain is a proteolytic enzyme. It has been reported to be present in cores, peels, flesh, stems and leaves of pineapple [2]. Two distinct types of pineapple bromelain are well known: fruit bromelain (EC 3.4.22.33) which is the major proteolytic component in pineapple fruits, and stem bromelain (EC3.4.22.32), the major proteolytic cysteinyl protease in pineapple stems [3]. Unlike crude stem bromelain which is used widely in industry, application of fruit bromelain is very limited despite the large quantities of waste pineapple fruit portions at pineapple canneries [4].

The current commercial bromelain is usually extracted from the stem and immature fruit of the pineapple [5]. Conventionally the enzyme was extracted from pineapple juice by ammonium sulfate precipitation [2]. At present, new technologies have been used to advance the purity of bromelain, such as ultrafiltration, chromatograph and reverse micellar extraction [2, 6, 7].

Bromelain is used in food, chemical and medicine industry. In medicine field, bromelain has been reported to be effective in blood coagulation, reduction of tumor cell growth and increase in cellular immune response [8, 9]. In addition, bromelain has been found to display anti-inflammatory activities [10-12] and be effectiveness in treating postoperative edema after third molar surgery [13]. In the food industry, bromelain has been reported that bromelain was effective in decomposing the structure of wheat glutenin IgE-binding epitope to produce hypoallergenic wheat flour [14, 15]. It also could be used for meat tenderization, dietary supplement, protein hydrolysates production, marine product processing and baking [16].
In this study, the effects of fruit bromelain on baked biscuits were studied. The bromelain was first isolated from pineapple juice by precipitation and then purified through chromatography approach. The purified bromelain was added into dough before baking of biscuit. The aroma and flavor compounds of biscuits with and without addition of bromelain was analyzed by HS-SPME combined with GC-MS. The results may enhance our knowledges of the molecular mechanism behind taste and nutritional value changes with addition of bromelain in baking products.

2. Materials and Methods

2.1. Materials
Pineapples of Bali variety were purchased from an open market for fresh farm products in Zhanjiang in January and February. Fresh pineapples were used for each biological repeat. L-cysteine, casein and ethylene diamine tetra acetic acid (EDTA) were obtained from Sigma. Dialysis membrane with molecular-weight-cut-off of 8,000 Da was obtained from Spectrum Medical Industries. SP-Sepharose Fast Flow and Sephadex G100 were made available from GE Healthcare. All other reagents were of analytical grade or best grade available.

2.2. Preparation of bromelain extraction
Bromelain was extracted from pineapple juice which was extracted from pineapple fruits by a commercial squeezer (JYZ-V2) and stored at 4 °C prior to use. Ammonium sulfate ((NH4)2SO4) was added to 5 L pineapple juice with gentle stirring until its saturation reached 60%. After incubation overnight at 4 °C, the suspension was centrifuged at 8000 rpm for 15 min [17]. The pellet at the bottom of the centrifuge tubes was collected and dissolved in 50 mM acetate buffer pH 3.5 and dialyzed against the same buffer for 24 hours at 4 °C.

The dialyzed enzyme solution was loaded onto a cation-exchange column (SP Sepharose Fast Flow) and a Sephadex G100 column successively both of which were pre-equilibrated with the 50 mM acetate buffer whose pH was 3.5. Elution was performed with a linear gradient of 0-0.1 M sodium chloride in 50 mM acetate buffer pH 3.5 at 2 ml/min, and 1 ml fractions were collected. The fractions with bromelain activity were pooled together and concentrated to around 5 ml by freeze-drying.

Protein concentration in the enzyme solutions was determined by the Bradford method using bovine serum albumin as a standard [18]. Activity of bromelain was assayed using casein as substrate in the presence of EDTA [19]. The hydrolysis reaction was conducted at 37 °C and pH 6.5 for 10 min. One unit of bromelain activity was defined as 1 μg L-tyrosine released in 1 min by one gram (or milliliter) of enzyme sample (U). Purity of extracted bromelain was analyzed by protein electrophoresis according Laemmli [20].

2.3. Preparation of biscuits
The biscuits were prepared according to Baljeet et al. protocol [21]. Briefly, the ingredients composed of 80 g refined wheat flour, 20 g buckwheat flour, 60 g sugar and 35 g butter, 1 g baking powder, 30 ml milk, 0.2 g salt, 0.5-1% (v/w) bromelain solution. Biscuits without addition of bromelain were made as control. Butter and sugar were mixed together using with a flat beater for 2 min at slow speed. The resulting cream was mixed well with flour, milk, salt and/or bromelain in dough mixer and keep still at room temperature for 30 min. The batter was then shaped and baked at 1900 °C for 12 min in a baking oven. The biscuits were cooled at room temperature and stored in fridge for further analysis.

2.4. Determination of the aroma and flavor compounds
The aroma and flavor compounds of the biscuit were determined by using headspace solid phase microextraction (HS-SPME) combined with GC-MS (PE, USA), according to the report described by Kataoka [19].

2.5. Statistical analysis
Standard mass spectra version of Nist08 and Wiley9 were used to identify and calculate the relative content of each compound with three biological repeats.

3. Results and Discussion

3.1. Production of bromelain
After purification and concentration, bromelain activity in final solution was $1.15 \times 10^6$ U/ml. The purity of the bromelain obtained was confirmed using SDS–PAGE (Figure 1). The molecular size of the extract was around 26.50 kDa, which was consistent with the research conducted by Rowan AD et al. [22].

![SDS–PAGE of bromelain with different loading amount](image1)

Figure 1. SDS–PAGE of bromelain with different loading amount

3.2. Effect of bromelain on the aroma and flavor compounds of the biscuit
The aroma and flavor compounds and their relative contents in the biscuit were analyzed by using HS-SPME combined with GC-MS. Total ion chromatogram (TIC) of the aroma and flavor compounds in the biscuit were showed in figure 2.

![Total ion chromatogram of the aroma and flavor compounds](image2)
Figure 2. TIC of the aroma and flavor compounds in the biscuit with addition of different amount of bromelain. A: control, B: 0.5% of bromelain (v/w), C: 1.0% of bromelain (v/w).

The major aroma and flavor compounds in the control were 12.07% 2-isobutyl-4,4-dimethyl-1,3-dioxane, 12.50% cyclotrisiloxane, 12.31% cyclotetrasiloxane and 7.64% cyclopentasiloxane (Table I). With addition of bromelain, the aroma and flavor compounds varied and their content changed. This may due to that the hydrolysis of dough protein by bromelain impacted the biochemical procedure of baking.
Table 1. Identified aroma and flavor compounds and their relative contents in the biscuit with different supplement of bromelain

| Compounds                             | Relative content (%) | Supplement of bromelain |
|---------------------------------------|----------------------|--------------------------|
| 2-Isobutyl-4,4-dimethyl-1,3-dioxane   | 12.07                | 23.65                    |
| Furan                                 | 1.11                 | 6.85                     |
| Cyclotrisiloxane                      | 12.50                | 5.01                     |
| 1-Hexanol                             | 0.53                 | 0.15                     |
| Benzaldehyde                          | 2.03                 | 3.92                     |
| 1-Octen-3-ol                          | 0.75                 | 0.86                     |
| Cyclotetrasiloxane                    | 12.31                | 0.84                     |
| 1-Octanol                             | 0.24                 | 0.41                     |
| Nonanal                               | 0.98                 | 1.60                     |
| Cyclopentasiloxane                    | 7.64                 | 0.31                     |
| Dodecane                              | 0.24                 | 0.29                     |
| Decanal                               | 0.39                 | 0.34                     |
| Heptasiloxane                         | 0.30                 | 0.19                     |
| Benzene                               | 0.18                 | 0.22                     |
| Cyclohexasiloxane                     | 6.44                 | 1.51                     |
| Heptadecane                           | 2.93                 | 0.96                     |
| 4-Morpholineethanamine                | 2.54                 | 2.05                     |
| Pentasiloxane                         | 2.48                 | 1.21                     |
| Cyclopentanemethylamine               | 0.17                 | 1.82                     |
| Phenol                                | 1.18                 | 1.43                     |
| Morpholine                            | 1.29                 | 1.00                     |
| Pentadecane                           | 1.51                 | 0.66                     |
| Hexadecane                            | 0.28                 | 0.36                     |
| n-Butylamine-D9                       | 3.89                 | -                        |
| L-Alanine                             | 1.03                 | -                        |
| 2-Propyl-1-pentanol                   | 0.35                 | -                        |
| Benzy1 Alcohol                       | 0.63                 | -                        |
| 2-Octen-1-ol                          | 0.16                 | -                        |
| Benzoic acid                          | 0.45                 | -                        |
| Phenylethyl Alcohol                   | 0.53                 | -                        |
| 1,3-Diphenyl-5-methylthio-1,2,4-triazole | 0.31             | -                        |
| 1H-Indene                             | 0.20                 | -                        |
| 7-CHLORO-1,3-DIHYDRO-5-PHENYL-         | 1.27                 | -                        |
| 4-AMINO-5-IMIDAZOLE CARBOXAMIDE       | 0.17                 | -                        |
| 1,6-Methano annulene                  | 0.20                 | -                        |
| 1-Butanol                             | 0.22                 | -                        |
| 3,3,5-Triethoxy-1,1,1,7,7,7-          | 0.49                 | -                        |
| Tetradecane                           | 0.90                 | -                        |
| Octadecanoic acid                     | 2.41                 | -                        |
| 1H-3a,7-Methanoazulene                | 1.64                 | -                        |
| Methyl {Di(trimethylsilyl)[dimethyl   | 0.44                 | -                        |
| Cedrol                                | 1.78                 | -                        |
| 1,4-Dioxaspiro[4,6]undecan-7-ol       | 0.77                 | -                        |
| 2,6,10-TRIMETHYLPENTADECANE           | 0.69                 | -                        |
| Cyclooctasiloxane                     | 0.91                 | -                        |
N,N-bis[2-Trimethylsiloxyethyl] ethanamine 1.30 -
2-Bromotetradecane 0.22 -
1-Propanol - 1.48
Ethanesulfonyl fluoride - 8.12
Ethanediimidic acid - 2.67
1,3-Benzodioxole-5-methanol - 0.74
Acetic acid - 0.66
D-Mannitol - 3.50
Manganese - 1.02
1-Propene - 1.72
Methyl-d3 1-Dideuterio-2-propenyl Ether - 2.18
Trimethylsilyl fluoride - 1.05
Benzenemethanol - 4.95
Cyclooctyl alcohol - 0.18
Octanoic acid - 0.37
11-Bromoundec-1-ene - 0.85
2,6-di-butyl-2,5-cyclohexadiene-1,4-dione - 0.20
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol - 0.63
2,6-DIHYDROXYBENZOIC ACID 3TMS - 0.44
2,8,9-Trioxa-5-aza-1-silabicyclo(3.3.3)undecane - 0.80

4. Conclusion
Bromelain could alternate the aroma and flavor compounds of biscuits, leading to the changes in their taste and nutritional value. The positiveness of these changes needs further study by sensory and nutrition evaluation. To our knowledge, this is the first study on the effect of bromelain on the aroma and flavor compounds of baked food, and thus these experiments are fundamental considering the further application of bromelain for using in baked food technology.

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