パイナップル由来酵素処理によるオレンジアレルゲンCit s 2濃度の減少

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著者名	清田,恭平 吉光,真人 梶村,計志 山野,哲夫
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Note

Reduction of Orange Allergen Cit s 2 Levels in Fresh Orange Juice with Pineapple Bromelain Enzymatic Treatment

Kyohei KIYOTA*, Masato YOSHIMITSU, Keiji KAJIMURA and Tetsuo YAMANO

Osaka Institute of Public Health:
1–3–69 Nakamichi, Higashinari-ku, Osaka 537–0025, Japan;
* Corresponding author

Oranges are consumed worldwide; however, they contain Cit s 2, a major profilin allergen. We aimed to reduce Cit s 2 levels by preparing mixed orange fresh juice with pineapple, as a convenient method for any kitchen. Cit s 2 levels in orange extracts digested with pineapple extract and its protease bromelain were evaluated with quantitative enzyme-linked immunosorbent assay. Cit s 2 levels decreased according to reaction temperature and time, which was inhibited by iodoacetic acid. Treatment with pineapple extract diluted 40-fold and 0.1 mg/mL of bromelain at 37℃ for 30 min contributed to reducing residual Cit s 2 levels below the cut-off of 15%, respectively. Since this condition can increase the proportion of orange juice and reduce the risk of ingesting the pineapple allergen bromelain, it is considered to be more practical. Broad utilization of proteases in hypoallergenic food products is expected following clinical studies for verification.

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Key words: orange profilin; Cit s 2; pineapple bromelain; allergen digestion

Introduction

Oranges are one of the most consumed fruits in the global market, offering rich nutrition with various health benefits[1), 2)]. Oranges are also used in several popular products such as juices, jams, and ice creams. In particular, fresh orange juice is widely consumed because of the ideal balance of acidity, sweetness, and fragrance. However, oranges can also cause food allergies similar to well known allergen-containing foods such as eggs, milk, and wheat[3)], but frequency of causing food allergy is low. Since there is currently no treatment for food allergy, individuals with known allergies must be cautious to regularly avoid allergen ingestion. In recent years, new enzymatic digestion methods have been developed for controlling the allergenicity of causative food allergens[4), 5)]. Enzymatic treatment is a simple and effective technique to reduce the allergen ingestion risk, which can be easily introduced to food processing steps, and has been demonstrated in various applications, using plant, animal, or microbial proteases.

Fresh juices from various fruits and vegetables are often mixed, which expands the array of juice products available. Orange juices are particularly congenial with many other types of fruits such as pineapple, kiwifruit, and papaya. These fruits commonly contain cysteine proteases that play various roles in the biological activities of plants, such as flower development, responses to environmental stress, and programmed cell death[6)]. The cysteine protease of pineapple, namely bromelain, has powerful activity as an endoprotease[7)], and has thus been widely used as a processing aid for meat tenderization or as a medical component for the treatment of inflammatory conditions[8)]. In addition, one study suggested the potential of bromelain to digest food allergens[7)].

Therefore, in this study, we investigated whether bromelain could reduce the allergen levels in orange. For this purpose, we focused on the major orange allergen profilin Cit s 2[9)], which causes oral allergy syndrome[10)]. We used a previously developed ELISA for quantifying Cit s 2[11)] to evaluate the degree of reduction of Cit s 2 levels in orange achieved by simply preparing a mixture of fresh orange juice with pineapple juice or purified bromelain as a widely accessible process.

Materials and Methods

1. Sample preparation

Samples of navel orange (*Citrus sinensis*) and pineapple (*Ananas comosus*) were purchased from local supermarkets in Osaka City, Japan. After washing the fresh fruits with water and drying with a paper towel, the pulp and peel of the navel orange and the pulp of the pineapple were homogenized with a domestic mill (IFM-800DG, Iwatani Co., Tokyo, Japan). The homogenates were shaken horizontally at 190 rpm for 15 min and extracted with water (1 : 1, w/v), followed by centrifugation at 8,000 $\times g$, 10℃ for 10 min. The extracts were stored at −20℃ until further analysis. Recombinant Cit s 2 (rCit s 2) and poly-L-proline affinity-purified native Cit s 2 (nCit s 2) was prepared as previously reported[11)]. rCit s

*kiyota@iph.osaka.jp

2 was used as a standard for Cit s 2-ELISA and as the spiking agent in the recovery test.

2. Orange extract digestion

Orange extracts were digested with pineapple extracts or purified bromelain (>800 units/mg, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) under the following conditions. Forty microliters of orange extracts were mixed with 40 μL of the enzyme solutions; pineapple extracts and pineapple extracts diluted 40-fold with water or with 4.0 mg/mL and 0.1 mg/mL of bromelain (final concentrations of 1.78 and 0.04 mg/mL at the reaction concentration, respectively). Furthermore, 1) 10 μL of water or 2) 10 μL of 180 mmol/L of the bromelain irreversible inhibitor iodoacetic acid (IAA; final concentration 20 mmol/L) was added; this concentration was chosen based on a previous study[12]. The temperature was set to 4℃, 25℃, and 37℃, and the reaction time was set to 5, 10, and 30 min. After the reaction, to stop bromelain enzymatic activity, the digests were immediately placed on ice, and 10 μL of 180 mmol/L of IAA was added to the digests of 1). The same volume of water was added to the digests of 2). To denature the enzyme, pineapple extracts and purified bromelain were heated at 90℃ for 20 min and used to digest Cit s 2 at 37℃ for 30 min. Finally, all of the digested solutions were stored at −20℃ until further Cit s 2 quantification. Each digestion was performed in three independent experiments.

3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

In order to verify the digestion of the Cit s 2, 9 μL of 500 μg/mL of nCit s 2 was treated with 3) 2 μL of 10 mg/mL of purified bromelain and 2 μL of water or 4) 2 μL of 10 mg/mL of purified bromelain and 2 μL of 1.8 mol/L of IAA at 50℃ for 2 h, respectively. After the reaction, 2 μL of 1.8 mol/L IAA was added to 3) and the same volume of water was added to 4). The same quantities of nCit s 2 as a control were treated at 50℃ for 2 h, followed by addition of the same volume of 1.8 mol/L IAA.

The samples were denatured at 95℃ for 5 min and then separated on SDS-PAGE gels (NuPAGE™ Novex™ 12% Bis-Tris protein gels, Thermo Fisher Scientific, Waltham, MA, USA) for 35 min at 200 V in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (Thermo Fisher Scientific). After electrophoresis, gel was stained with Coomassie brilliant blue (Bio-safe CBB G-250 stain, Bio-Rad Laboratories, Hercules, CA, USA).

4. Cit s 2 levels evaluation

The validated quantitative Cit s 2-ELISA was used to evaluate Cit s 2 levels as reported previously with small modifications[11]. In brief, flat-bottomed 96-well microplates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were incubated overnight at 4℃ with 50 μL/well of 5 μg/mL anti-Cit s 2 monoclonal antibody (mAb) 38-1 diluted in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6). The plates were blocked for 1 h with phosphate buffered saline (PBS) containing 20% horse serum (Life Technologies) and washed three times with PBS containing 0.05% Tween 20 (PBST). PBST was used in all subsequent washing procedures. The samples for Cit s 2-ELISA were prepared by diluting the digest solution with PBST containing 20% horse serum (1 : 3, 1 : 9, or 1 : 14 v/v). Tween 20 was added to prevent non-specific binding between proteins. After adding 25 μL/well of the biotin-labeled anti-Cit s 2 mAb 47-1 (1 : 2,000), an equal volume of the sample or standard rCit s 2 was added to the same well. Following incubation for 1 h, the plates were washed five times, and then 50 μL of streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) was added to the wells. Following incubation for 1 h and washing the plates eight times, 3,3′,5,5′-tetramethylbenzidine (Thermo Fisher Scientific) was added. The color reaction was performed for 10 min in the dark and was stopped by adding 50 μL of 1 mol/L H_2SO_4. The absorbance measurement at 450 nm and data analysis was carried out with a FlexStation 3 microplate reader and SoftMax Pro 7.0.3 software (Molecular Devices, CA, USA), respectively. The serial dilution calibration curve was generated using rCit s 2 at the range of 0.63–40 ng/mL with the lower limit of quantification (LOQ) at 2.5 ng/mL. All measurements were performed in duplicate.

5. Calculation of residual Cit s 2

After stopping the orange allergen digestion reaction, the residual Cit s 2 in the orange was calculated as follows:

$$\text{Relative percentage of residual Cit s 2 (\%)} = C_{S1} \times 100/C_1,$$

where C_{S1} is the concentration of the orange extract under any treatment at 37℃ for 30 min and C_1 is the concentration of the orange extract treated with water as a blank at 37℃ for 30 min, as determined by Cit s 2-ELISA, respectively.

$$\text{Percentage of residual Cit s 2 (\%)} = C_{S2} \times 100/C_2,$$

where C_{S2} is the concentration of the orange extract under any treatment and C_2 is the concentration of the orange extract treated with the same conditions without reaction time, as determined by Cit s 2-ELISA, respectively.

6. Cit s 2 recovery test

After the digestion of the orange extracts with pineapple extracts or 4.0 mg/mL of purified bromelain at 37℃ for 30 min and adding the bromelain inhibitor, we spiked 10 ng/mL of rCit s 2 to the digested solutions. The percentage recovery of rCit s 2 was then calculated as the means of three independent experiments as follows:

$$\text{rCit s 2 recovery (\%)} = (C_{S3} - C_3) \times 100/10,$$

where C_{S3} is the concentration of the spiked solution and C_3 is the concentration of the non-spiked solution, as determined by Cit s 2-ELISA, respectively.

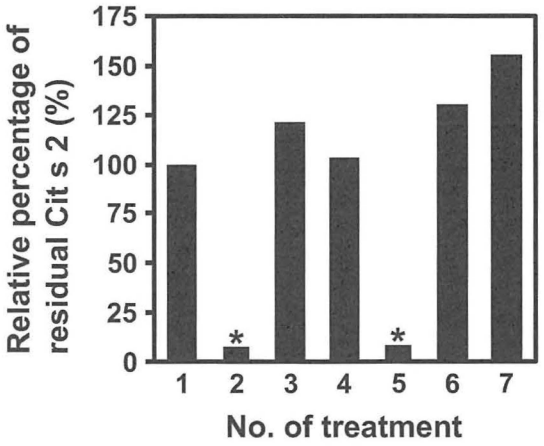

Fig. 1. Relative percentage of residual Cit s 2 in orange extract

Treatment at 37℃ for 30 min with no. 1, water as a blank; no. 2, pineapple extract; no. 3, pineapple extract and IAA as an inhibitor; no. 4, pineapple extract denatured at 90℃ for 20 min; no. 5, 4.0 mg/mL of bromelain; no. 6, 4.0 mg/mL of bromelain and IAA; no. 7, 4.0 mg/mL of bromelain denatured at 90℃ for 20 min. The bar graph shows average values of percentages ($n=6$; 2 tests × 3 determinations each). * The value was below the lower limit of quantification in Cit s 2-ELISA.

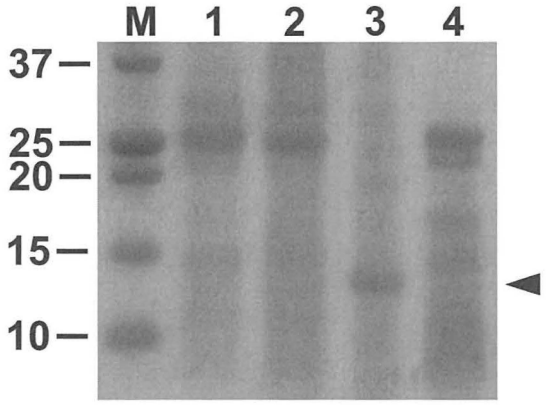

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

A band of nCit s 2 was observed at an approximate molecular weight of 14,000 (black arrowhead). Lane 1, nCit s 2 treated with purified bromelain; lane 2, nCit s 2 treated with purified bromelain and IAA; lane 3, nCit s 2; lane 4, purified bromelain; and M, molecular weight marker (× 10^3). Lanes 1–3 contained 4.5 μg of nCit s 2 and lanes 1, 2, and 4 contained 20 μg of purified bromelain, respectively.

Results and Discussion

1. Inhibition of bromelain activity and validation of Cit s 2-ELISA

In this study, we focused on the potential of using the pineapple-derived protease bromelain to digest Cit s 2, the major allergen of orange, since pineapple is one of the most suitable fruits for combining with orange juice. Although food protein hydrolysates potentially contain bitter peptides[4], which may negatively affect the flavor and consumer preference of processed foods, a mixture of orange and pineapple juice can improve the taste and palatability. Furthermore, both pineapple fruit and purified bromelain are widely commercially available[13].

Indeed, the Cit s 2 level was clearly reduced after treatment with pineapple extracts and 4.0 mg/mL of purified bromelain at 37℃ for 30 min, and this reduction was suppressed by adding 20 mmol/L of the bromelain inhibitor IAA or denaturing pineapple extracts and bromelain at 90℃ for 20 min (Fig. 1). The recovery rate of rCit s 2 in the digested solutions was acceptable with both the pineapple extract and 4.0 mg/mL of purified bromelain at 119.6% and 106.0%, respectively. In addition, no Cit s 2 was detectable by ELISA (<LOQ) after digestion with pineapple extract or purified bromelain as a negative control. This finding confirmed the deactivation of bromelain, and validated the measurement of Cit s 2-ELISA.

2. SDS-PAGE analysis

Next, we optimized the reaction conditions for Cit s 2 digestion and performed SDS-PAGE analysis (Fig. 2). The band of nCit s 2 was observed at the approximate molecular weight of 14,000 in lane 3, while no such band was observed in lane 1, because of the digestion with bromelain enzyme. In lane 2, the band of nCit s 2 treated with bromelain enzyme and IAA was unclear. This was considered as follows: since IAA, as an alkylating agent, reacted with nCit s 2 and the smear proteins of bromelain enzyme such as observed at a molecular weight of 20,000 or less in lane 4, the complicated smear bands overlapped with the band of nCit s 2.

3. Reduction of Cit s 2 levels after digestion

Since our main goal is to develop an effective method of allergen reduction that can be easily introduced to general food processing with a thermal sterilization stage (under 85℃ for 30 min or equivalent conditions[*1]) and cooking routines, we used simple and practical conditions for the digestion procedures: extracting with water rather than a buffer and omitting an optimized pH adjustment for enzymatic activity, although the pH of orange and pineapple extracts is similar (approximately 4.0). Furthermore, to avoid losing the flavors and nutrients of the juice by heating at high temperature, the reaction temperature to digest Cit s 2 was set at 4, 25, and 37℃, corresponding to a refrigerator, kitchen, and body temperature and hot water at kitchen sink, respectively.

To determine the minimum dilution rate contributing to the residual levels of Cit s 2 below 15% (the minimum cut-off value to remain above the LOQ) at 37℃ for 30 min, we tested various dilution ratios of pineapple extracts and determined a 40-fold dilution to be suitable.

[*1] Japanese Ministry of Health, Labour and Welfare, https://www.mhlw.go.jp/content/000420821.pdf

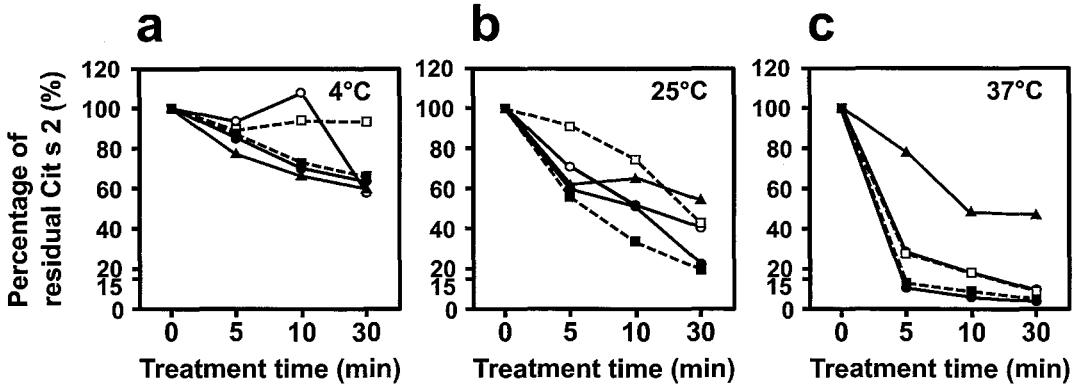

Fig. 3. Percentage of residual Cit s 2 in orange extract

Treatment at (a) 4℃, (b) 25℃ and (c) 37℃ for 5, 10, and 30 min with the pineapple extract (solid line and black circles), the pineapple extract diluted 40-fold (solid line and white circles), 4.0 mg/mL of purified bromelain (dotted line and black squares), 0.1 mg/mL of purified bromelain (dotted line and white squares), and water as a blank (gray line and black triangle). Average values of percentages are plotted ($n = 3$).

Addition of pineapple extracts and pineapple extracts diluted 40-fold to orange extracts reduced Cit s 2 levels in a manner dependent on both the reaction temperature and time (Fig. 3). Considering that pineapple extracts contain approximately 0.4% bromelain[14], the same amount of purified bromelain was tested directly, at 4.0 mg/mL and 0.1 mg/mL, respectively, and had the same effect on reducing Cit s 2 levels as the pineapple extracts (Fig. 3). Cit s 2 levels in orange extracts treated with water as a blank tended to reduce, because the diluted and lowered concentration of Cit s 2 may be instable. The reduction of Cit s 2 levels was suppressed by adding 2 × PBST containing 40% horse serum instead of water during the reaction (data not shown). Although treatment at 4℃ was better for food preservation, the digestive effect couldn't be expected because of low temperature. Treatment at 25℃ for 30 min with pineapple extracts and 4.0 mg/mL of purified bromelain archived a less than 25% of reduction of Cit s 2 levels in orange. From the perspective of digestion processing time and completeness, treatment at 37℃ for 10 min with pineapple extract and 4.0 mg/mL of purified bromelain was considered to be the most effective condition. Furthermore, treatment at 37℃ for 30 min with pineapple extract diluted 40-fold and 0.1 mg/mL of purified bromelain is considered to be more practical, because of the smaller amount of enzyme required and the increase of the relative amount of orange juice to keep the orange flavor.

4. Future tasks for promotion of Cit s 2 digestion with bromelain

Since heat-processed orange juices may have residual allergenicity of Cit s 2[15], enzymatic digestion may be a key process to produce hypoallergenic orange products. The results of the present study demonstrate the potential hypoallergenicity of Cit s 2 using pineapple extract and purified bromelain, although the outcome requires clinical verification in further study. Since there is no detailed information regarding human IgE epitopes of Cit s 2, we do not know if Cit s 2-ELISA recognizes the IgE epitopes. Nonetheless, this study is a primary approach for orange allergen reduction, because there are only few orange allergen analysis methods[11]. We will need to test the orange allergen reduction using immunoblotting or ELISA with the serum of orange allergic patients in future.

However, it is also important to consider that bromelain itself is a pineapple allergen, namely Ana c 2[16]. Since the amino acid sequence identity between Cit s 2 and Ana c 2 is very low, individuals with orange allergy who react to Cit s 2 may not cross-react to bromelain. Nonetheless, the potential cross-reactivity between orange and pineapple should be interpreted with caution. This is because another pineapple allergen, Ana c 1, is a profilin family protein that is highly conserved among fruit plants, and has high amino acid sequence identity with Cit s 2. Considering that the optimum pH and temperature for pineapple fruit bromelain varies according to the substrates such casein and hemoglobin, i.e., pH 2.9–7.7 and 37–59℃[7], the reaction conditions for Cit s 2 digestion should be further optimized. This will help to reduce the amount of bromelain needed in orange juice, and therefore decrease the risk of pineapple allergens ingestion, although this comes at a cost of slightly more complicated food process steps. Overall, it is recommended to declare pineapple on product labels so as to provide reliable information for consumers.

Proteases are widely used in the food industry, and thus their application to allergen reduction is expected. Future studies of protease-based allergen reduction methods will lead to improvement of the QOL of individuals with food allergy and their families who share their diet.

In conclusion, we demonstrated that enzymatic treatment at 25℃ achieved a certain degree of reduction of Cit s 2 levels in orange, which was promoted by heating up to 37℃. The treatment of 37℃ for 30 min with a smaller volume of pineapple extract diluted 40-fold and 0.1 mg/mL of purified bromelain allowed for achieving a

residual Cit s 2 level below 15%, which is considered to be more practical. This simple treatment method can increase the proportion of orange juice in the final product while reducing the ingestion risk of the pineapple allergen bromelain, Ana c 2. Further expansion of the application of proteases in addition to bromelain in the production of hypoallergenic food products is expected. Therefore, future studies to confirm these hypoallergenic effects are warranted for the benefit of individuals with food allergy and their families.

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パイナップル由来酵素処理によるオレンジアレルゲン Cit s 2 濃度の減少（ノート，英文）

清田恭平 *，吉光真人，梶村計志，山野哲夫

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オレンジは，健康に有益な栄養成分を含む一方で，アレルギーの発症原因となるアレルゲンも含んでいる．オレンジアレルギーの発症を予防するためには，アレルゲンの摂取リスクを抑えることが重要である．そこで本研究では，果物ミックスジュースにおいて，オレンジとの組合せで嗜好性のよいパイナップルに含まれるタンパク質分解酵素ブロメラインの利用に着目した．パイナップル由来酵素を利用して，オレンジの主要アレルゲンである Cit s 2 の濃度減少が可能かどうか，Cit s 2 定量 ELISA により評価を行った．生鮮オレンジ果汁に対して生鮮パイナップル果汁を添加したところ，Cit s 2 濃度は反応の時間や温度に依存して減少する傾向が見られた．特に，オレンジ果汁に対し 1/40 量のパイナップル果汁を添加して 37℃30 分間処理した場合，Cit s 2 濃度が 15％未満（定量下限値未満）に減少した．今後，慎重な臨床的検証が必要であるものの，オレンジアレルゲン低含有量の果物ミックスジュースの調理・製造方法として，本研究の応用が期待される．

* 大阪健康安全基盤研究所