Formation of Protein-Bound 3,4-Dihydroxyphenylalanine and 5-*S*-Cysteinyl-3,4-dihydroxyphenylalanine as New Cross-Linkers in Gluten

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The formation of protein-bound 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine (5-*S*-cysteinyldopa) and 3,4-dihydroxyphenylalanine (dopa) as new cross-linkers in gluten was investigated. Gas-phase acid hydrolysis of gluten was carried out in the presence of 5% phenol. For identification of dopa and 5-*S*-cysteinyldopa, alumina extraction of catechols in the hydrolysates was performed. The presence of dopa and 5-*S*-cysteinyldopa in the hydrolysates of gluten was determined using HPLC equipped with an electrochemical detector. When mushroom tyrosinase, which has an oxidant-like effect on the physical properties of dough, was added to dough, the amount of protein-bound 5-*S*-cysteinyldopa increased. It is concluded that the oxidizing effect of tyrosinase is due to the formation of protein-bound 5-*S*-cysteinyldopa and dopa as cross-linkers of gluten.

Keywords: *5-S-cysteinyl-3,4-dihydroxyphenylalanine (5-S-cysteinyldopa); 3,4-dihydroxyphenylalanine (dopa); gluten; tyrosinase*

INTRODUCTION

In rheological properties of dough, disulfide groups can cause interchange reactions with thiol groups. During dough mixing, only 1−2% of all gluten disulfide bonds can be broken by exchange with free sulfhydryl groups (Mauritzen, 1967), indicating that only a few reactive disulfide bonds are crucial to rheological properties. Jones et al. (1974) concluded that 48−56 μmol of SH residues/100 g of flour (only 25−35% of total SH content) affected farinograph dough development and mixing tolerance. Less than 4% of the total disulfide is involved in determining development time of dough, and 11−13% is involved in the resistance to mixing (Jones et al., 1974). About 25−35% of the total thiol is involved in both development and tolerance to mixing (Jones et al., 1974).

Bread dough is a highly complex chemical system. While covalent and ionic bonds primarily increase cohesiveness of doughs, dipole, hydrogen, and hydrophobic bindings contribute to its elasticity and plasticity. Van der Waals interactions are apparently of limited significance. Such a diversity of forces complicates interpretation of factors that regulate dough structure. It seems that practically all types of bonds contribute to the dough formation (Pomeranz, 1987).

In addition to the postulated interchange reaction, thiol groups may react with other compounds besides disulfides by oxidation.

Recently, simple thiol compounds were shown to form complexes with quinones or oxidized products. Several investigations have shown that oxidation products of phenol compounds conjugate with SH compounds (Finley, 1974). The conjugation of dopa with cysteine is also mediated by tyrosinase, peroxidase−H_2O_2, superoxide radical, hydroxyl radical, and iron−EDTA complex (Ito and Prota, 1977; Ito et al., 1984; Ito and Fujita, 1984; Ito, 1983; Kato et al., 1986).

Nishiyama et al. (1979) postulated that the addition reaction between flour SH groups and mushroom enzyme-oxidized phenols in flour is responsible for the enhancing effect of mushroom extract on dough. It can be postulated that tyrosinase catalyzed the addition between oxidized tyrosine and SH groups contribute such an effect on mushroom extract on dough. Ito et al. (1984) demonstrated that mushroom tyrosinase can catalyze hydroxylation of tyrosine residues in proteins to dopa and subsequent oxidation to depaquinone residues. The dopaquinone residues in proteins combine with cysteine residue to form 5-*S*-cysteinyldopa in bovine serum albumin and yeast alcohol dehydrogenase. Thus, such complexes may contribute to the cross-linking of gluten but have yet to be identified in doughs.

Whether oxidized tyrosine moieties in gluten react with SH groups, causing changes in the physical properties of dough, would be of great interest.

We report here new types of cross-linkers, protein-bound 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine or 3,4-dihydroxyphenylalanine, in gluten.

MATERIALS AND METHODS

Reagents. Activated alumina and mushroom tyrosinase (activity 1800 units/mg) were obtained from Wako Pure Chemical Industries Ltd. (Osaka). All other reagents were of guaranteed grade and commercially available. Authentic 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine was provided by Prof. Shosuke Ito (Fujita Health University).

Preparation of Gluten. The flour used was commercially milled No. 1 Canada Western Red Spring provided by Showa Sangyo Co. Ltd., with a 12.8% protein content and 0.43% ash content. Doughs were prepared with 200 g of flour and 120 g of water in a mixer (SD-BT2, National). Doughs were mixed for 20 min in the presence of air. In the experiments with added tyrosinase, 4 or 8 mg of commercial mushroom tyrosinase was added to 200 g of flour, for the modification of the

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physical properties of dough (Kuninori et al., 1979). Doughs were incubated for 4 h at 28 °C. Gluten was washed in a stream of tap water until the wash water was clear and then freeze-dried, ground, and stored at −20 °C.

Gas-Phase Amino Acid Hydrolysis of Gluten. The freeze-dried glutens were hydrolyzed with vapor using 6 N HCl containing 5% phenol at 105 °C for 24 h, according to the method described by Kato et al. (1995). Before hydrolysis, the container was evacuated for 10 min and saturated with N_2 gas. The treatment was repeated three times, and the container was evacuated for 10 min, sealed, and then hydrolyzed under the conditions mentioned above. The hydrolysate was concentrated in vacuo and dissolved in 200 μL of 0.01 M HCl.

HPLC. Separation was achieved on a Develosil ODS-HG-5 column (4.6 × 250 mm, Nomura Chemical Co., Ltd.) with a solution of 10 g of phosphoric acid, 7 g of methanesulfonic acid, and 0.1 mmol of Na_2EDTA per liter of water. The eluent's pH was adjusted to 2.35 with 5 M soldium hydroxide/methanol (19:1), and an isocratic flow of 0.8 mL/min was maintained. The eluent was monitored with an electrochemical detector (ECD; JASCO 840-EC) set at +750 mV (electrode potential). The peak area was determined using a JASCO 807-IT integrator. The eluted dopa was detected by a fluorometric method (excitation = 280 nm; emission = 320 nm) using a fluorescence spectrometer (JASCO FP-920).

Alumina Extraction. Alumina extraction of catechols in the hydrolysates was performed according to the method described by Kato et al. (1986). To the dried hydrolysates of gluten was added 200 μL of 6 N HCl. Ten microliters of 10 μg/mL α-methyldopa in 0.1 M HCl was added as an internal standard. Catechols in the hydrolysates were absorbed onto alumina (pH 8.5) by adding 50 mg of alumina, 100 μL of 2% $Na_2S_2O_5$, and 1 mL of 2.7 M Tris/2% Na_2EDTA, and the mixture was immediately shaken for 5 min on a multitube mixer. The alumina was washed three times with about 1 mL of water, and then catechols were eluted with 0.3 mL of 0.4 M $HClO_4$. The extract was analyzed by HPLC as described above. The dopa and 5-*S*-cysteinyldopa values in this paper were corrected for recovery of an internal standard.

RESULTS AND DISCUSSION

Formation of Dopa and 5-*S*-Cysteinyldopa. The formation of dopa has been reported by some workers as one of the oxidized products of the tyrosine residue (Kato et al., 1995; Gieseg et al., 1993). The formation of protein-bound dopa and 5-*S*-cysteinyldopa as cross-linkers in gluten was investigated. The chromatograms of acid hydrolysates of gluten are shown in Figure 1. A product was detected in the hydrolysates at a retention time of 9.2 min (Figure 1a), which was comparable with that of authentic dopa. Dopa was confirmed in the gluten by the described alumina absorption and subsequent HPLC analysis (Figure 1b). The absorbed product was observed at the same position as authentic dopa. The eluted dopa was detected by fluorometry (excitation = 280 nm; emission = 320 nm) using a fluorescence spectrometer (FP-920). The mixture of authentic dopa and catechols extracted from acid hydrolysates of gluten was injected and showed a single peak in HPLC. One of the catechols was then identified as dopa.

The ECD retention times of authentic 5-*S*-cysteinyldopa were compatible with a product of hydrolysates. Another catechol in acid hydrolysates of gluten was observed at the same position as authentic 5-*S*-cysteinyldopa as shown Figure 1c. A co-injection of catechols extracted from acid hydrolysates of gluten and authentic 5-*S*-cysteinyldopa provided a single peak. The product was then identified as 5-*S*-cysteinyldopa.

Kato et al. (1986) reported HPLC analysis of protein-bound cysteinyldopas. Contrary to their work, we did not find 2-*S*-cysteinyldopa, 2,5-di-*S*-cysteinyldopa, and

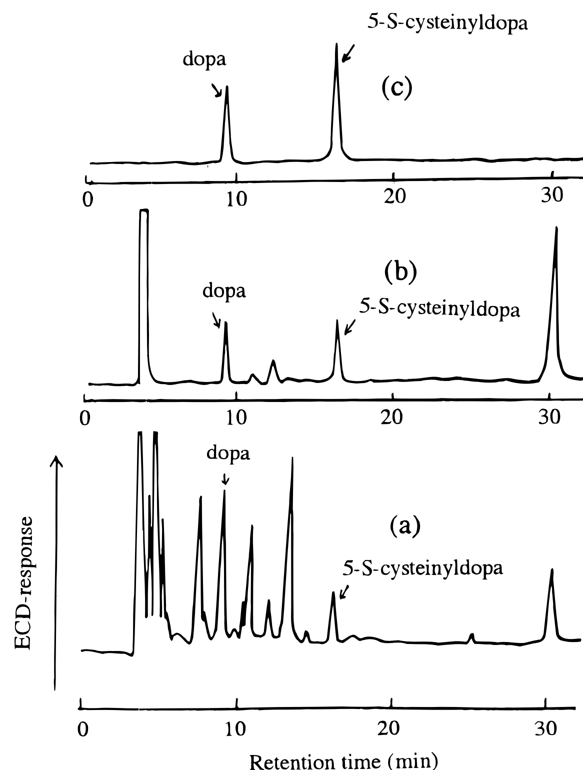


Figure 1. HPLC chromatograms of acid hydrolysates of gluten: (a) acid hydrolysate of gluten; (b) catechols in the acid hydrolysate of gluten; (c) authentic dopa and 5-*S*-cysteinyldopa.

6-*S*-cysteinyldopa in the hydrolysates of gluten after alumina extraction.

Dopa can be generated enzymatically using mushroom tyrosinase, which catalyzes the hydroxylation of tyrosine residues (Ito et al., 1984). Dopa is also a major reductant formed during hydroxyl radical damage to protein (Gieseg et al., 1993). Covalent adduct formation was reported between dopaquinone and amino acids such as lysine or histidine (Waite, 1990; Schaefer et al., 1987). The conjugation of dopa with cysteine is also mediated by tyrosinase, peroxidase−H_2O_2, superoxide radical, hydroxyl radical, and iron−EDTA complex (Ito and Prota, 1977; Ito et al., 1984; Ito and Fujita, 1984; Ito, 1983; Kato et al., 1986).

The acid hydrolysates of our freeze-dried gluten had 283 nmol of dopa and 26.7 nmol of 5-*S*-cysteinyldopa per 100 g of flour, confirming that protein-bound dopa and 5-*S*-cysteinyldopa are formed in gluten during mixing in the presence of air. The mechanism of formation of protein-bound dopa and 5-*S*-cysteinyldopa is shown in Scheme 1. The mechanism of the reaction between SH groups and oxidized tyrosine residue in gluten should be examined.

Effects of Mushroom Tyrosinase on Formation of Dopa and 5-*S*-Cysteinyldopa. Formation of dopa and 5-*S*-cysteinyldopa in the hydrolysates of gluten is shown in Figure 2. Formation of 5-*S*-cysteinyldopa increased in response to mushroom tyrosinase quantity, but formation of dopa did not increase linearly when more mushroom tyrosinase was added. These results indicated that dopa was very reactive and was conjugated rapidly with cysteine residues. In our experiment, it was concluded that addition of mushroom tyrosinase to flour prior to dough formation is related to the formation of protein-bound 5-*S*-cysteinyldopa and dopa in gluten.

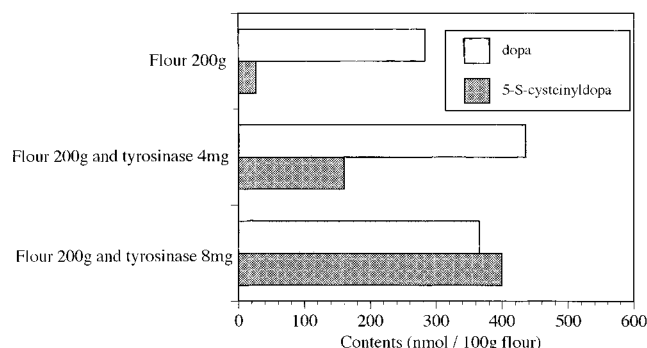
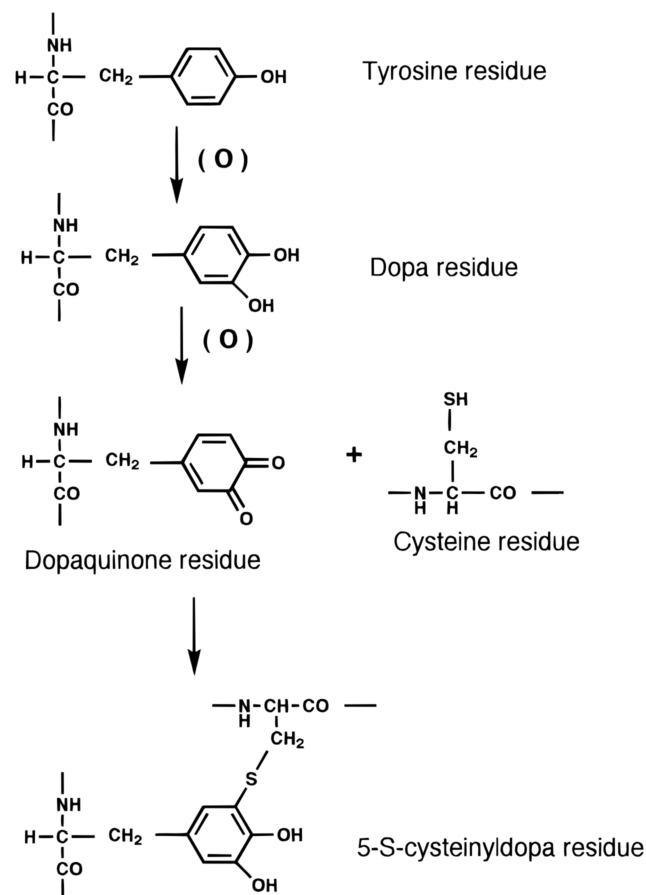


Figure 2. Effects of tyrosinase on formation of dopa and 5-*S*-cysteinyldopa in the hydrolysates of gluten. The data are presented as mean of two separate gluten determinations.

Scheme 1. Mechanism of Formation of Protein-Bound 5-*S*-Cysteinyldopa in Gluten



Kuninori et al. (1976) reported that dough-improving effects of mushroom extract may depend on tyrosinase. When SH−gluten or glutathione was incubated with the mushroom-oxidized digested gluten, a pronounced loss in the sulfhydryls was observed. None of this loss was caused by oxidation, since no increase in disulfides was detected (Nishiyama et al., 1979). These results indicate that other types of conjugation besides SS linkage exist in gluten and affect the physical properties.

Our results, considered in combination with those of Kuninori et al. (1976) and Nishiyama et al. (1979), suggest that the improving effect of mushroom extract on dough is due to the formation of protein-bound 5-*S*-cysteinyldopa in gluten. Such cross-linkings are expected to enhance the gluten network prepared with flour containing high levels of tyrosinase activity.

The presence of polyphenol oxidase (PPO) in wheat and wheat products has been well established (Milner and Gould, 1951). Marsh and Galliard (1986) studied PPO activity in wheat-milling fractions. PPO activity in white flour (four commercial samples), whole meal, and bran, determined by O_2 uptake on addition of catechol to aqueous suspensions of finely ground (<0.5 mm) materials, was 10−30, 420, and 640 nmol of O_2 min^{-1} g^{-1}, respectively. Hatcher and Kruger (1993) reported that the bran flours and shorts duster contained notably higher PPO levels than the other flour streams, using Canadian Common Wheat Classes milled to three extraction rates (about 70, 80, or 85%).

White flours contain relatively low PPO levels, suggesting that glutens prepared with whole meal are expected to contain higher levels of protein-bound dopa and 5-*S*-cysteinyldopa than white flour.

We showed that protein-bound 5-*S*-cysteinyldopa cross-links existed in gluten. It is considered that this conjugation can lead to polymerization of the proteins and/or other protein, though the amount of formation is low. Further study is necessary to elucidate the contribution of protein-bound 5-*S*-cysteinyldopa as a cross-linker in dough rheology.

ABBREVIATIONS USED

PB-dopa, protein-bound 3,4-dihydroxyphenylalanine; PB-5-S-CD, protein-bound 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine; 5-*S*-cysteinyldopa, 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine; dopa, 3,4-dihydroxyphenylalanine; ECD, electrochemical detector; PPO, polyphenol oxidase; HPLC, high-performance liquid chromatography; EDTA, ethylendiaminetetraacetic acid.

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