Riboflavin-Binding Protein Is a Novel Bitter Inhibitor

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Abstract

Riboflavin-binding protein (RBP) from chicken egg, which was recently reported to be a selective sweet inhibitor for protein sweeteners, was also found to be a bitter inhibitor. RBP elicited broadly tuned inhibition of various bitter substances including quinine-HCl, naringin, theobromine, caffeine, glycyl-L-phenylalanine (Gly-Phe), and denatonium benzoate, whereas several other proteins, such as ovalbumin (OVA) and β-lactoglobulin, were ineffective in reducing bitterness of these same compounds. Both the bitter tastes of quinine and caffeine were reduced following an oral prerinse with RBP. It was found that RBP binds to quinine but not to caffeine, theobromine, naringin, and Gly-Phe. However, the binding of RBP to quinine was probably not responsible for the bitter inhibition because OVA bound to quinine as well as RBP. Based on these results, it is suggested that the bitter inhibitory effect of RBP is the consequence of its ability to interact with taste receptors rather than because it interacts with the bitter tastants themselves. RBP may have practical uses in reducing bitterness of foods and pharmaceuticals. It may also prove a useful tool in studies of mechanisms of bitter taste.

Key words: bitter inhibitor, caffeine, quinine, riboflavin-binding protein

Introduction

Bitter tasting substances are generally avoided because they are often toxic. However, beer, coffee, and tea are exceptions to this general rule, perhaps, because they are characterized by prominent positive pharmacologic activity. Nevertheless, excessive bitterness of most foods must be avoided or they will not be consumed, particularly by children. Thus, there is a general goal in the food and pharmaceutical industry to develop debittering agents.

Several techniques for debittering have been developed such as use of bitter inhibitors (Breslin and Beauchamp 1995; Katsuragi et al. 1995; Ley et al. 2006), treatments with enzymes to degrade the bitter compound (Tan et al. 1993; Puri et al. 1996; Izawa et al. 1997), and entrapment of bitter substances from the product (Vaks and Lifshitz 1981; Lin et al. 1997).

During our study on the contributions of food proteins on the taste of food, riboflavin-binding protein (RBP) purified from hen egg white was found to be a selective inhibitor for protein sweeteners (Maehashi et al. 2007). RBP purified from chicken egg white and yolk exhibits a significant suppressive effect on protein sweeteners such as thaumatin, monellin, and lysozyme but does not show an inhibitory effect on other sweeteners of small molecular size such as sucrose, saccharin, and glycine. RBP is found in chicken egg white at 0.09% (Rhodes et al. 1959) and binds riboflavin tightly in a 1:1 molar ratio. Therefore, it is a source of riboflavin to the developing embryo. Since first isolated from hen's egg white by Rhodes et al. (1958), the structure of RBP has been well studied. RBP is a monomeric phosphorylated glycoprotein consists of 219 amino acid residues with 9 disulfide bonds (Hamazume et al. 1987). There have been many reports on RBP concerned with its riboflavin-binding activity. In addition to its sweet protein suppressing activity, we accidentally found that RBP may also be a potent suppressor of bitterness (Maehashi et al. 2007). Other bitter inhibitors, such as sodium salts (Breslin and Beauchamp 1995), lipoprotein (Katsuragi et al. 1995), flavanones (Ley et al. 2005), and benzylamides (Ley et al. 2006), are currently used in industry. However, a natural protein-based bitter inhibitor might prove useful for many food applications where excessive bitterness is an issue. In this paper, we examine the characteristics of bitter inhibition of RBP.

Materials and methods

Materials

Fresh White Leghorn eggs were obtained from a local food market. Quinine hydrochloride, theobromine, denatonium

benzoate, bovine serum albumin (BSA), and ovalbumin (OVA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Naringin, caffeine, and β-lactoglobulin (LG) were purchased from Sigma-Aldrich (Tokyo, Japan). Glycyl-L-phenylalanine (Gly-Phe) was purchased from Peptide Institute, Inc. (Osaka, Japan). DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, and Sephadex G-25 were purchased from Amersham Biosciences Corp. (Piscataway, NJ).

Purification of RBP from hen egg white and yolk

Purification of apo-form RBP from chicken egg white and yolk was performed essentially according to the method of Miller and White (1986) with minor modifications as described in our previous paper (Maehashi et al. 2007). Chicken egg white collected from 40 eggs was diluted twice and added to 300 ml of buffered DEAE-Sepharose CL-6B (0.1 M acetate buffer, pH 4.3) gel to entrap RBP. After the gel was packed into a column, RBP was eluted with a linear NaCl concentration gradient of 0–1 M in the buffer. Then the yellow fraction was subjected to ammonium sulfate fractionation at 55–85% $(NH_4)_2SO_4$ saturation. The yellow precipitate was collected and subjected to CM-Sepharose CL-6B (buffered with 25 mM acetate buffer at pH 3.14). After the yellow fraction was eluted, the column was washed with 25 mM acetate buffer (pH 5.8) to obtain the apo-form RBP and then it was subjected to gel filtration on a Sephadex G-25 column to remove the acetate salt.

Hen egg yolk collected from 40 eggs was diluted with an equal volume of 0.1 M acetate buffer (pH 4.3) and centrifuged at $9000 \times g$ for 1 h. The supernatant was dialyzed against deionized water for 24 h and then against 0.1 M acetate buffer (pH 4.3) for 24 h. The same procedure as used for egg white was then conducted to obtain yolk RBP. Both RBPs, isolated through chromatography, were dialyzed against deionized water to remove the buffer salt and then lyophilized.

Dephosphorylation of RBP

RBP was dephosphorylated with acid phosphatase as described by Miller et al. (1982) and McClelland and Price (1998). Egg white RBP (300 mg) was dissolved in 30 ml of 0.1 M sodium acetate buffer (pH 5.3), and 1 ml of potato acid phosphatase (Wako Pure Chemical Industries), dissolved with 1% BSA solution at 60 units/ml, was added. This mixture was dialyzed against 1 l of buffer in a water bath at 37 °C for 3 h. The dephosphorylated proteins were isolated from the reaction mixture by chromatography on Sephadex G-25 (0.1 NaCl/25 mM sodium acetate, pH 5.3), DEAE-Sepharose CL-6B column (0.1 M sodium acetate, pH 5.3, gradient of 0–1 M NaCl), and Sephadex G-25 column (deionized water) in order. The dephosphorylated RBP was then dialyzed and lyophilized.

Measurement of bitter compounds bound to protein

Bitter compound solutions and mixtures of the solution of the bitter compound and protein (dissolved in distilled water, at room temperature) were filtered with Microcon YM10 (10 000 MW cut-off; Millipore Corp., Billerica, MA) by centrifugation. Concentrations of the bitter compound in both filtrates were measured with a Beckman DU640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at the wavelengths of absorption maximums, which were in advance determined from the results of UV spectrum (200–400 nm) for each bitter compounds. A percentage of the bitter compound in the filtrate of the mixture against that in the filtrate of bitter compound alone was calculated as unbound (%).

Determination of equi-bitter concentrations to 0.125 mM quinine for various bitter compounds

Bitter compounds were dissolved in deionized water, and their equi-bitter concentrations to 0.125 mM quinine were determined by paired comparisons test using 6 well-trained judges (4 females and 2 males, average age = 22 years) from our laboratories. Bitter compound solutions were prepared at several concentrations, and the bitterness intensity of each solution was compared with that of 0.125 mM quinine solution which was of moderate bitterness at room temperature to all the judges. The tasting was conducted with rinses of mouth and a 3-min interval between samples.

Measurement of bitter inhibitory activity

The bitter inhibitory activity was assayed by 4 judges who were chosen from the 6 in the section described above. They were trained according to the procedure described in this section. To measure bitter inhibitory activity of sample, at first 0.5 ml of a bitter substance solution at room temperature was held in mouth for 30 s to evaluate its bitterness and then expectorated. Following the evaluation, the mouth was rinsed repeatedly with deionized water. After 3 min, the bitterness disappeared totally, a solution of same bitter substances mixed with a sample of a potential inhibitor dissolved in deionized water was tasted. Its bitterness was compared with the first bitter compound solution that did not contain the potential inhibitor. Bitterness intensities were recorded using a 10-cm unstructured line scale with anchor points "Not at all" and "Equi-bitter to that without potential inhibitor." For more bitter than it, the intensity was recorded beyond anchor point on the elongate line. Inhibition was represented as the percentage of the intensity of bitterness of the bitter compound with the potential inhibitor compared with the bitter intensity without the potential inhibitor.

Results and Discussion

Effect of RBP on bitterness of various bitter substances

In previous paper (Maehashi et al. 2007), we found that RBP exhibited not only selective inhibition toward protein sweeteners but also inhibited the bitterness of quinine. As shown in Figure 1, RBP inhibited bitterness of 0.125 mM quinine, whereas it did not inhibit the sweetness of 0.15 M sucrose, the

saltiness of 0.15 M sodium chloride, the umami quality of 25 mM monosodium glutamate, or the sourness of 10 mM citric acid. Thus, RBP can be said that a selective inhibitor for quinine bitterness. Bitter inhibition of RBP toward various other bitter compounds were evaluated next. In order to study the effect of RBP on each bitter compound at approximately the same level of bitter intensity, the 4 judges first determined the concentration of each bitter compound that is equivalent to the moderate bitterness of 0.125 mM quinine. The results are listed in Table 1, From these results, concentrations of each bitter compounds for the test of bitter inhibition by RBP were selected as indicated in Figure 2. Some bitter compounds used were chosen as representatives of bitter principles found in food, that is, naringin for grapefruits, caffeine for coffee, Gly-Phe for protein hydrolysates (a model for fermented food), and theobromine for cacao. Denatonium benzoate and quinine hydrochloride were chosen as represents of drugs.
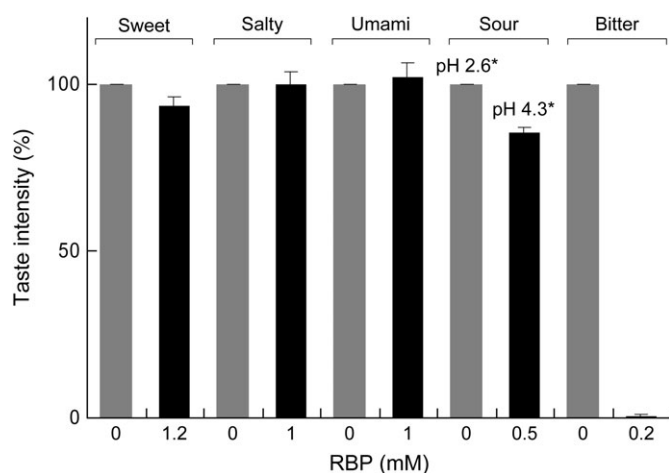

Figure 1 Effect of RBP on various taste stimuli. Sucrose (0.15 M), 0.15 M sodium chloride, 25 mM monosodium glutamate, 10 mM citric acid, and 0.125 mM quinine were used as standard solutions for sweetness, saltiness, umami, sourness, and bitterness stimuli, respectively. The taste intensity of these compounds with added RBP was compared with the intensity of the same compound without added RBP expressed as a percentage. pHs were measured for citric acid. Vertical bars indicate standard errors.

Table 1 Equi-bitter concentrations of bitter compounds to 0.125 mM quinine[a]

Bitter compounds	Concentration
Naringin	1.1 ± 0.1 mM
Denatonium[b]	0.1 ± 0.01 µM
Theobromine	6.5 ± 0.4 mM
Caffeine	13.7 ± 0.3 mM
Gly-Phe	57.5 ± 3.2 mM

[a]Quinine hydrochloride.
[b]Denatonium benzoate.

As shown in Figure 2, the bitterness of all bitter compounds tested was inhibited by RBP in a concentration-dependent manner. Even when we increased the concentration of bitter quinine to 0.25 and 0.5 mM (200% and 400%, respectively), 1 mM RBP completely suppressed the bitterness (Figure 3).

Unlike our previous study on RBP suppression of the sweetness of protein sweeteners (Maehashi et al. 2007), selectivity was not observed in bitter inhibition of RBP. Because RBP showed a broadly tuned bitter inhibition toward structurally diverse compounds in this experiment, it was suggested the possibility of practical use of RBP as a novel inhibitor for bitterness of foodstuffs and pharmaceuticals.

Structural specificity of bitter inhibition by RBP

Perhaps, all proteins are capable of masking bitterness of foodstuffs. To examine the specificity of RBP as the bitter inhibitor, 3 commercially available proteins, OVA, LG, and BSA were compared with RBP for their effect on the bitterness of 0.125 mM quinine or 1 mM naringin. None of the proteins tested in this experiment had any taste themselves. As shown in Figure 6, the bitterness of 0.125 mM quinine was not altered by 0.5 mM concentrations of OVA, LG, or BSA, whereas it was inhibited completely by RBP even at lower concentrations than the other proteins. Furthermore, the bitterness of 1 mM naringin was not altered by 1 mM of these 3 commercially available proteins, but it was inhibited by 1 mM RBP. These results indicate that bitter inhibition is not a common attribute of all proteins; instead, it is
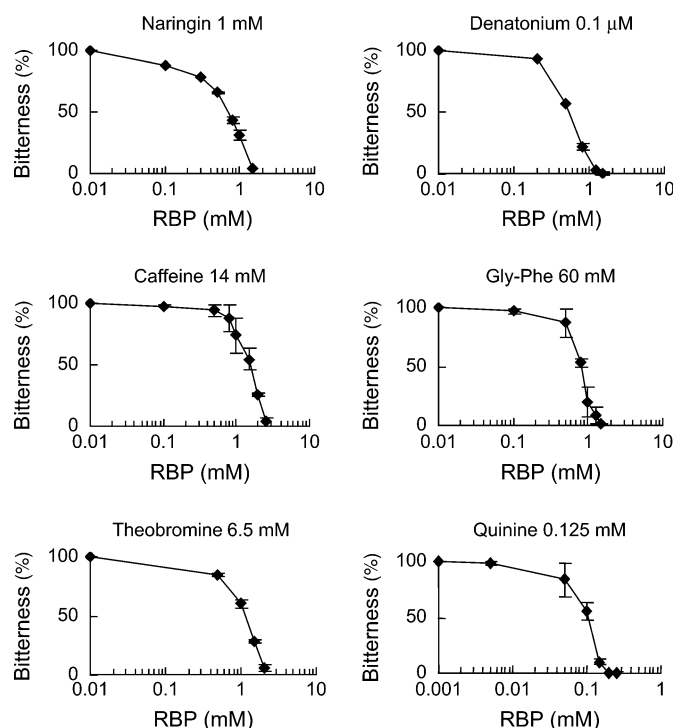

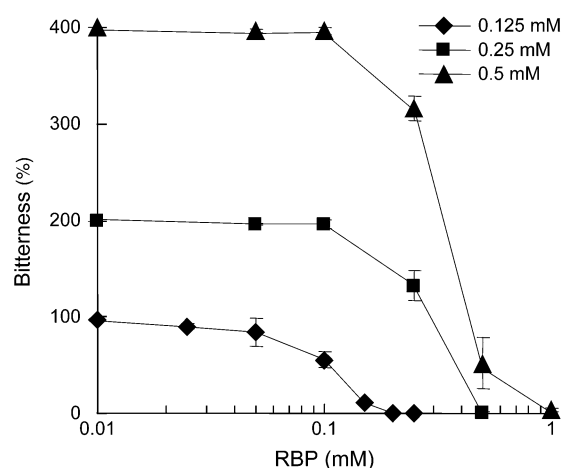**Figure 2** Effect of RBP on the bitterness of various bitter compounds.

Figure 3 Effect of RBP on the bitterness of quinine at multiple concentrations.

Figure 4 Bitter inhibition of RBPs with structural difference. W-RBP, egg white RBP; Y-RBP, egg yolk RBP; Dephospho-RBP, dephosphorylated RBP.

lane 1: native RBP
lane 2: dephospho-RBP

Figure 5 Native polyacrylamide gel electrophoresis (PAGE) analysis of dephosphorylated RBP and native RBP. Native PAGE was performed using a 4–15% gradient polyacrylamide gel (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) under nondenaturing condition. The gel was stained with Coomassie brilliant blue R-250.

a relatively unique property of RBP. Katsuragi et al. (1995) showed that the bitterness of 0.5 mM quinine was greatly suppressed by 3% lipoprotein composed of phosphatidic acid (PA) and LG and 0.85% of PA liposome but hardly at all by 2.15% of LG alone. They suggested that complex formation with PA was required for LG to suppress bitterness. Our results were consistent with their results in that LG did not suppress bitterness. RBP is the first reported protein that inhibits bitterness. RBP binds one molar of riboflavin in its molecule at the position between Tyr75 and Trp156 (Monaco 1997). Because no difference was detected in the activity of the apo- and holo-forms of RBP (data not shown), it was considered that riboflavin itself does not elicit bitter inhibition and also the structure of the riboflavin-binding site within RBP molecule does not participate in this activity. Therefore, the apo-form of RBP was used for all experiments in this study.

RBP is found not only in egg white but also in egg yolk. The differences between the 2 RBPs are the C-terminal amino acid sequence and the structure of oligosaccharide chains (Norioka et al. 1985). It is also known that egg yolk RBP has the same characteristics of structure as white RBP, such as the positions of the carbohydrate chains and the phosphate groups in the sequence (Hamazume et al. 1984). Therefore, egg white and yolk RBP were compared for their effect on bitterness. Whereas white RBP inhibited the bitterness of 0.125 mM quinine completely at 0.2 mM, yolk RBP inhibited only about 50% of the bitterness at 0.2 mM; complete suppression was observed at 1 mM (Figure 4). This difference of bitter inhibition may be due to the difference of C-terminal structure or to the oligosaccharide chain between them. However, because both RBPs inhibited bitterness, the C-terminal structure of RBP is not essential for bitter inhibition.

The effect of RBP phosphate groups of RBP was examined next. Dephosphorylated RBP was obtained by treatment with acid phosphatase followed by isolation from the reaction mixture. As shown 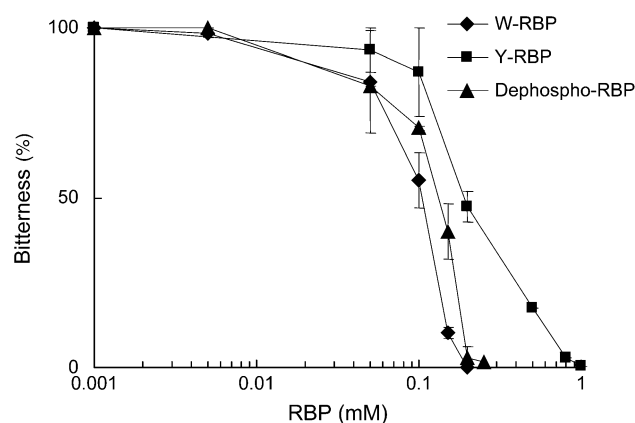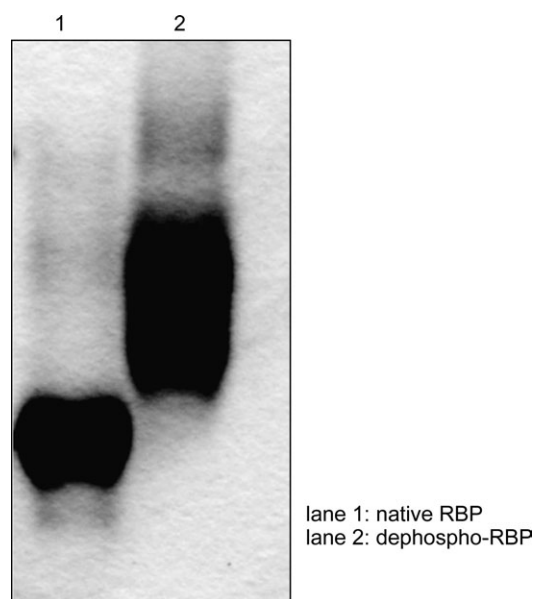in Figure 5, dephosphorylated RBP was confirmed to be different in its electric charge from that of native RBP. However, the effect of dephosphorylated RBP on bitterness of 0.125 mM quinine was almost the same as native RBP, showing complete inhibition at 0.2 mM (Figure 4). This result indicates that the negative charge of phosphate groups of RBP does not participate in bitter inhibition.

Effect of prerinse with RBP on the bitterness

To further characterize bitter inhibition by RBP, we examined the effect of an oral prerinse with RBP solution on the bitterness of 0.125 mM quinine and 14 mM caffeine. After
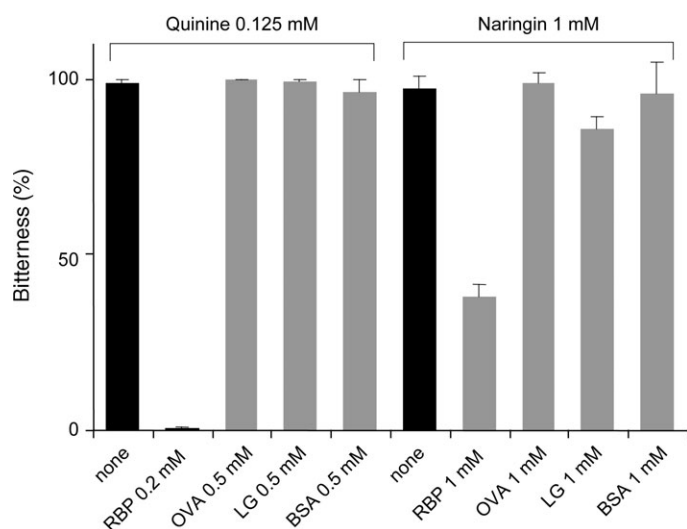
Figure 6 Effect of proteins on the bitterness of quinine and naringin. RBP, riboflavin-binding protein.

Figure 7 Effect on bitterness following a prerinse with RBP. Both 0.125 mM quinine and 14 mM caffeine were tasted after the mouth was exposed to a 2 mM RBP solution for 20 s. "−," without prerinse; "+," following prerinse.

0.5 ml of 2 mM RBP solution was held in the mouth for 20 s and expectorated, each bitter solution was immediately evaluated for bitterness intensity compared with the taste of the bitter solutions without a prerinse of RBP but with a water rinse. As shown in Figure 7, after a rinse with 2 mM RBP, the bitterness intensity of 0.125 mM quinine and 14 mM caffeine was significantly reduced. This result was similar to the result of PA-LG by Katsuragi et al. (1995) that treatment of tongue with 3.0% PA-LG decreased the bitter taste of 0.5 mM quinine and 50 mM caffeine. Moreover, they showed adsorption of PA-LG on frog tongue surface and concluded that the PA-LG could bind to the receptor sites for bitter substances on the taste receptor membranes (Katsuragi et al. 1996). Our results indicate that the bitter inhibition is most likely caused by masking of the bitter receptor site on tongue surface with RBP as was suggested for PA-LG. However, it was not considered that RBP binds to tongue surface persistently because the prerinse effect with RBP almost disappeared 2 min after expectoration of RBP solution (data not shown), not as the sweet inhibition by a prerinse with gymnemic acid lasts for 30 min (Gent et al. 1999).

Molecular interaction between RBP and bitter compounds

Because there is still a possibility that the bitter inhibition was the result of direct interaction of the bitter compounds with RBP. Thus, the possible binding of the bitter compounds with RBP was examined next. To accomplish this, the bitter compound solution and the mixture solution with RBP were each filtered with 10 000 molecular weight (MW) cut-off filters by centrifugation, then the concentrations of bitter substances in both filtrates were compared. If the bitter compound binds RBP, the complex should not pass through the membrane. The results (Table 2) show that 14 mM caf-
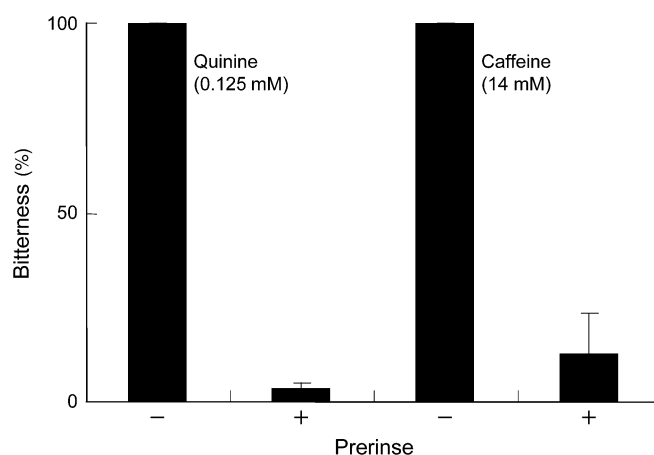
feine, 1 mM naringin, 6.5 mM theobromine, and 60 mM Gly-Phe, all of which were inhibited by 2 mM RBP (Figure 2), did not substantially bind to RBP, whereas quinine did significantly bind to RBP. Most of 0.125 and 0.25 mM of quinine was bound to 0.2 and 0.5 mM RBP, respectively. Interestingly, OVA, which did not exhibit bitter inhibition, bound to quinine as well as RBP. Moreover, only 53.9% and 14.6% of 0.25 mM quinine remained unbound after being mixed with 0.1 and 0.25 mM RBP, respectively, although they exhibited 98% and 66% of bitterness intensities of 0.25 mM quinine, respectively. These results indicate that the binding of quinine with protein does not correlate well with bitter inhibition. Instead, we suggested that masking on the tongue surface, probably at the level of the bitter receptor cells, is more likely to be responsible for the bitterness inhibition by RBP. Because RBP inhibited only the bitter taste modality and several other proteins did not alter bitterness, the interaction of RBP with receptor cells is rather specific. The result that quinine, which is known to be hydrophobic, was not detected in the filtrate of the mixture with RBP indicated that binding of RBP to quinine with hydrophobic interactions prevented quinine from having access to bitter receptors on taste cells. Katsuragi et al. (1995) suggested that PA-LG was effective in suppressing the bitter taste of quinine, promethazine, and brucine mostly due to the binding of these compounds to PA-LG by hydrophobic interaction. However, they proposed that the bitter suppressive effects on caffeine, glycyl leucine, and naringin are probably brought about by masking of target sites for bitterness on tongue with PA-LG. Based on our results that the bitterness of quinine was only inhibited by RBP, whereas quinine bound to both of RBP and OVA, it is suggested that the affinity of RBP to bitter receptor sites is higher than that of OVA to these receptor sites and that RBP binds to bitter receptor sites with a higher affinity than does quinine.

Table 2 Binding of bitter compounds with proteins

Bitter compound	Protein	Unbound (%)[a]
Quinine (0.125 mM)	RBP (0.2 mM)	0.0
	OVA (0.5 mM)	0.7
Quinine (0.25 mM)	RBP (0.1 mM)	53.9
	RBP (0.25 mM)	14.6
	RBP (0.5 mM)	1.5
	OVA (0.5 mM)	28.8
Caffeine (14 mM)	RBP (2 mM)	91.7
Naringin (1 mM)		89.5
Theobromine (6.5 mM)		69.2
Gly-Phe (60 mM)		98.8

[a]Unbound indicates the percentage of residual bitter compound in the filtrate of the mixture of bitter compound and protein after centrifugation using spin column with MW 10 000 cut-off membrane.

We proposed that the mechanism of bitter inhibition of RBP is a competition for binding to bitter receptor sites with bitter compounds, in case of quinine including the direct binding and sequestering of quinine. This seemed to be supported by the fact that bitterness of quinine was inhibited by lower concentration of RBP as compared with other bitter compounds (Figure 2).

Members of a family of approximately 30 divergent G protein–coupled receptors, termed T2Rs, probably function as detectors of bitter taste compounds (Chandrashekar et al. 2000). T2Rs are selectively expressed in the tongue and palate epithelium. Ligands for some of them have been reported. For example, human (h) T2R4 responds to denatonium (Chandrashekar et al. 2000), hT2R16 is a candidate receptor for β-glucopyranosides (Bufe et al. 2002), hT2R61 responds to 6-nitrosaccahrin (Pronin et al. 2004), hT2R14 is a candidate receptor for picrotoxin (Behrens et al. 2004), and hT2R44 and hT2R43 are receptors for denatonium, aristolochic acid, and 6-nitrosaccharin (Kuhn et al. 2004). That is, each bitter compound may have the highest affinity for a specific T2R receptor. However, the binding site for bitter receptors has not been determined. Probably there are several specific binding sites for each bitter compound in the T2R receptors. Recently, it is suggested that distinct taste receptor cells expressing T2Rs are bitter receptor cells and act as broadly tuned bitter sensors (Mueller et al. 2005; Chandrashekar et al. 2006). Because RBP exhibited broadly tuned bitter inhibition toward structurally diverse bitter substances but did not inhibit other taste modalities, it is likely that RBP interacts with bitter receptor proteins or bitter receptor cells with high affinity to inhibit access of any structurally diverse bitter substances to them. RBP may also serve as a specific probe that may be useful in labeling these bitter receptor cells.

Conclusions

RBP, a protein found in egg, functions as bitter inhibitor. RBP, which is tasteless itself, exhibited bitter inhibition that was broadly tuned for various bitter substances. Therefore, RBP may be useful for suppressing bitterness of pharmaceuticals and foodstuffs. This discovery implicates a novel function of food protein in reducing bitterness by its interaction with bitter receptor sites on the tongue surface. RBP should also be useful for studying bitter structure–activity relationships, and it may help in elucidating the mechanisms underlying bitter taste perception.

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