大麦・小麦α-およびβ-チオニンによる耐酸耐熱性細菌 Alicyclobacillus acidoterrestrisの制御

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Control of Thermoacidophilic *Alicyclobacillus acidoterrestris* by Barley and Wheat α- and β-Thionins

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Key words: thionin, *Alicyclobacillus acidoterrestris,* antibacterial peptide, fruit juice, thermoacidophilic bacteria

Contents

I Introduction

Recently, the spoilage of fruit juice by thermoacidophilic spore-forming bacterium *Alicyclobacillus acidoterrestris* has been observed worldwide. Fresh fruit juice and juice concentrates are often contaminated with spores of *A. acidoterrestris*[5),18)], and they have survived after pasteurization[21),25)]. The bacterium produces an off-odor compound, guaiacol[17),18)], but not any toxin in fruit juice.

The growth of *A. acidoterrestris* in fruit juice has been regulated by nisin which is an antibacterial peptide from *Lactococcus lactis* subsp. *lactis*[9),26)]. Sodium hypochlorite killed the spores[16)], and egg white lysozyme reduced the heat-tolerance of the spores[25)]. To prevent the spoilage of fruit juice by *A. acidoterrestris*, ultra high-temperature heat-treatment (UHT) is the most effective since this bacterium has not been found in any commercial UHT juice[18)]. However, the UHT usually spoils flavor of fruit juice. There-

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fore, the control of *A. acidoterrestris* by the combinational treatment of antibacterial substances from agricultural products and pasteurization are examined in this study.

Barley and wheat seeds contain peptides of α- and β-thionins, which consist of 45 amino acids and inhibit the growth of various microorganisms[6], but there are only a few reports on the inhibition to Gram-positive bacteria. In particular, the growth inhibition of *A. acidoterrestris* by the thionins has not been reported at all. The α- and β-thionins were also heat-stable[15] and extractable from barley and wheat flours by 0.05 N H_2SO_4[15] and by 0.05-0.2 N HCl[13]. Barley and wheat α- and β-thionins are also called as α- and β-hordothionins and α- and β-purothionins, respectively. Most fruit juices originally contain citric and malic acids, which are widely used as food additives. If the α- and β-thionins are inhibitive to *A. acidoterrestris* and are extractable by citric and malic acids from barley and wheat flours, the crude extracts containing α- and β-thionins may prevent spoilage of fruit juice by *A. acidoterrestris*. This report describes growth inhibition of *A. acidoterrestris* by the α- and β-thionins, extraction of the α- and β-thionins from barley grains by citric and malic acids, and control of *A. acidoterrestris* in fruit juice by the addition of the extracts.

Ⅱ Materials and methods

1 Purification of α- and β-hordothionins

α- and β-Hordothionins were purified from a barley cultivar "Ichibanboshi". The grains were milled by a Cyclotec 1093 sample mill with 1 mm φ mesh screen. Milled barley flour was washed twice in a 20 mM phosphate buffer (pH 7.2) containing 1 mM thiourea at 4℃ for 1 h. It was then extracted with the phosphate buffer containing 0.5 M NaCl for 2 h. The α- and β-hordothionins in the extracts were precipitated by adding crystal ammonium sulfate until a saturation of 50% to 90% was obtained. They were then purified by high-performance liquid chromatography (HPLC) with a column of Wakosil 5C4-200 (Wako, Osaka, Japan) using a gradient solvent system of 0-40% (0-50 min) acetonitrile containing 0.1% trifluoroacetic acid at 0.5 ml/min by monitoring of OD_{224}. Each fraction (0.5 ml) was dried out with a centrifugating evaporator. To prepare the stock solution, α- and β-hordothionins were dissolved with autoclaved distilled water. Portions of the stock solutions were re-analyzed by HPLC (Fig. 1).

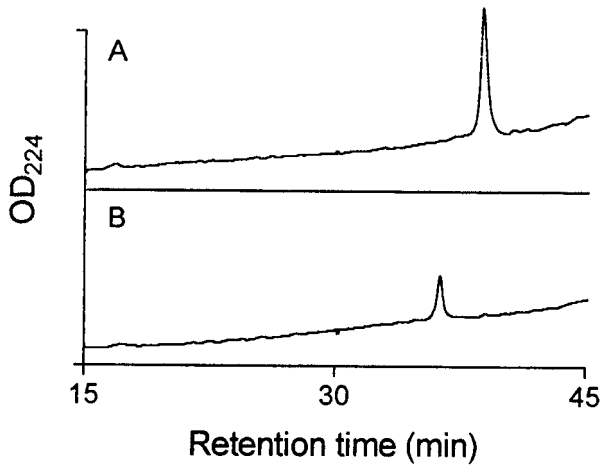

Fig.1 HPLC profiles of purified α- and β-hordothionins. A: α-hordothionin; B: β-hordothionin; column: Wakosil 5C4-200; gradient solvent: 20-40-50% (0-30-35 min) acetonitrile containing 0.1% trifluoroacetic acid.

2 Assay for antibacterial activity

A. acidoterrestris ATCC 49025 was pre-cultured at 37℃ for two days in a yeast-peptone-glucose broth (YPGB: yeast extract, 2.5 g; polypeptone, 5 g; D-glucose, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 2 g; distilled water, 1,000 ml) at pH 4[25]. The pre-culture was heated at 60℃ for 1 h to activate spores and inactivate vegetative cells[21]. A portion of the heated pre-culture containing 5×10^4 spores was inoculated into 1 ml of YPGB, which was supplemented with 1-100 μg/ml of purified α- and β-thionins. Purified α-purothionin was purchased from Takara Biochemical, Shiga, Japan. α-Purothionin is further classified to α1- and α2-purothionins[6]. The purchased α-purothionin

was identified as α1-purothionin by amino acids composition analysis[14]. The minimal inhibitory concentration (MIC) was defined as the minimal concentration at which no increase of OD_{590} in the broth was observed after two days of incubation at 37℃. The viable cells were indicated by colony forming unit (CFU). CFU was counted using potato dextrose agar (Wako, Osaka, Japan) (PDA) plates containing 0.1% DL-malic acid. Autoclaved PDA was mixed with a sterile malic acid solution prior to plating, and the pH of medium changed to around 3.8. Samples were diluted with a 150 mM NaCl solution and were spread on the PDA plates. Colonies on the plates were counted after three days of incubation at 37℃.

Peeled satsuma mandarin and an apple cultivar "Fuji" were homogenized by High Power Homogenizer II (Hirosawa Iron Works, Tokyo, Japan). After centrifugation (10,000 *g* for 1 min), the supernatants were used for the antibacterial assay. In the assay for antibacterial activity in fruit juice, *A. acidoterrestris* was pre-cultured at 37℃ for three days in fresh 100% satsuma mandarin juice (12.3° Brix, pH 3.5), 30% dilution of apple juice (4.2° Brix, pH 3.7), or commercial mixed fruit-vegetable juice (9.2° Brix, pH 4.2) containing juices of carrot and apple, etc. The strain ATCC 49025 did not grow in fresh 100% apple juice (13.7° Brix, pH 3.6), but grew in the 30% dilution of apple juice. The dilution of apple juice was prepared by diluted with distilled and autoclaved water. The pre-culture and fruit juices containing α- and β-thionins or barley extract were separately heated at 80℃ for 10 min[18] before inoculation. The juice in 2 tubes was inoculated with a portion of the pre-culture and was incubated at 37℃. The CFU was counted as shown above and the MIC was defined as the minimal concentration at which no increase of the average CFU after two days of incubation at 37℃.

3 Extraction of α- and β-hordothionins by citric and malic acids

Barley grains were milled by the same method in the purification of α- and β-hordothionins. Milled flours (1 g) were mixed with 4 ml of 0.05-0.2 M citric acid or 0.2 M DL-malic acid, and the mixtures were incubated for 30 min at 20℃ with 10 sec of bortexing every 10 min. After centrifugation at 2,000 *g* for 1 min, the supernatant (2.6ml) of the mixture was stored at -30℃ until it was added to the fruit juice. A portion (2.5 μl) of the extract was analyzed by SDS-PAGE and silver staining[14]. To compare the amount of α-hordothionin in the extracts, another portion (50 μl) of the extracts was fractionated by HPLC under the same conditions for the purification of α- and β-hordothionins. The fractions containing α-hordothionin were dried out by a centrifuging evaporator and were dissolved with 50 μl of distilled water. A portion (5 μl) of the sample was loaded to SDS-PAGE. The amount of extracted α-hordothionin was compared by area and density between silver-stained bands in the gels.

Ⅲ Results

1 Growth inhibition of *A. acidoterrestris* by purified α- and β-thionins

The MIC of α- and β-thionins against *A. acidoterrestris* in YPGB was 5 μg/ml for α-hordothionin and α-purothionin, and 10 μg/ml for β-hordothionin. α-Purothionin (20 μg/ml) reduced the CFU in YPGB from 5×10^4/ml to 1×10^3/ml for 6 h, and the reduced CFU was maintained for three days (Fig. 2). The CFU in the medium without α-purothionin was the highest after 24 hr of the incubation, but decreased after 48 and 72 hr of the incubation (Fig. 2). The pH of the medium after 72 hr of the incubation was 6.5, and the pH elevation was probably attributed to bacterial conversion of polypeptone to ammonia. The reduction of CFU may be caused by the neutral pH of the medium, because the growing pH range of *A. acidoterrestris* was 3.0-6.0[21].

The MIC of α- and β-hordothionins and α-purothionin against *A. acidoterrestris* in the satsuma mandarin juice was all 15 μg/ml. The reduction of the CFU by 20 μg/ml α-purothionin in three kinds of

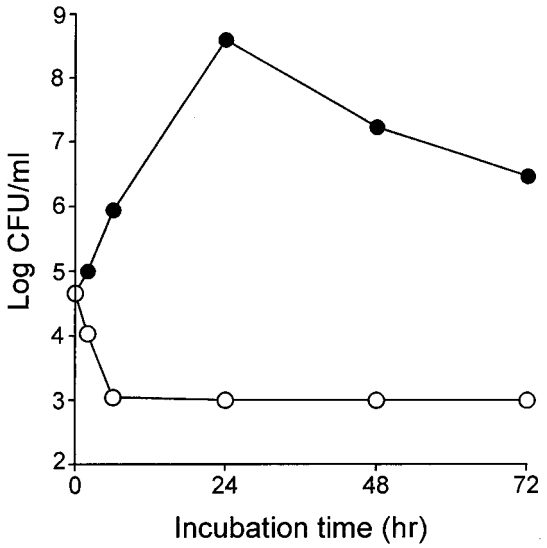

Fig.2 Growth inhibition of *A. acidoterrestris* by α-purothionin in YPGB. Open circles: 20 μg/ml α-purothionin; closed circles: no thionin.

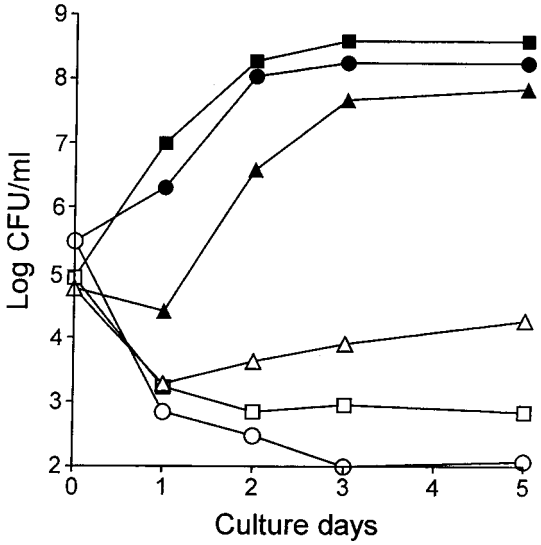

Fig.3 Growth inhibition of *A. acidoterrestris* by α-purothionin in fruit juice. Circles: satsuma mandarin juice; triangles: 30% dilution of apple juice ; squares: commercial fruit-vegetable juice mixture; open symbols: 20 μg/ml α-purothionin; closed circles: no thionin.

fruit juice is shown in Fig. 3. The CFU in the satsuma mandarin juice with α-purothionin decreased from 3×10^5/ml to 100/ml after a three-to-five-day incubation at 37℃. A similar inhibition was shown in the mixed fruit-vegetable juice, but the inhibition was relatively weak in the 30% dilution of apple juice. The viable cells in the dilution of apple juice containing α-purothionin decreased after one day of incubation, but they gradually increased for two-to-five days.

2 Extraction of α- and β-hordothionins by citric and malic acids

α- and β-Hordothionins were extracted from barley flour by 0.2 M citric acid and malic acid (Photo. 1). Since the difference of molecular weights between α-hordothionin and β-hordothionin was only 41 Da[6], α- and β-hordothionins were detected at the same position in this gel. The citric and malic acid extracts seem to contain similar total amounts of α- and β-hordothionins. A concentration of citric acid higher than 0.2 M and/or an extraction time in excess of 30 min caused viscose extract, which seemed to be unsuitable for addition to fruit juice. These extracts also contained considerable amounts of proteins other than α- and β-hordothionins, and the protein composition was different between these extracts.

α- and β-Hordothionins in the extracts were separated by HPLC (Fig. 4) but the amounts of α- and β-

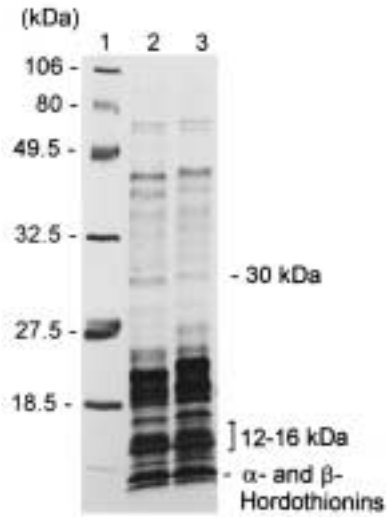

Photo.1 SDS-PAGE of barley extracts. Lane 1: marker proteins; 2: 0.2 M citric acid extract; 3: 0.2 M malic acid extract.

hordothionins were difficult to estimate by peak area because the baseline of the two peaks of α- and β-hordothionins is obscure. To estimate the amounts of α-hordothionin in the HPLC fractions, the fractions were analyzed by SDS-PAGE (Photo. 2). α-Hordothionin was detected in the HPLC fractions from the extracts of 0.5-0.2 M citric acid and of 0.2 M malic acid, but not of distilled water. These HPLC fractions were still contaminated with unknown 20 kDa proteins, and the band position of the proteins was slightly different between the extracts of citric and malic acids. The HPLC fraction of the 0.2 M citric extract contained larger amount of α-hordothionin than that of the 0.05 and 0.1 M citric acid extracts and almost equal amount of α-hordothionin compared with that of the 0.2 M malic acid extract.

3 Control of *A. acidoterrestris* in satsuma mandarin juice by barley extracts

The effect of the barley extracts with 0.2 M citric and malic acids on the growth of *A. acidoterrestris* in the satsuma mandarin juice was shown in Fig. 5. When the satsuma mandarin juice was inoculated with about 2×10^5 spores/ml of *A. acidoterrestris*, the average CFU after 2 days of incubation increased in the orange juice containing 1% and 2% of the citric acid extract but decreased in the juice containing 3% and 5% of the extract (Fig. 5A). The average CFU of *A. acidoterrestris* in the satsuma mandarin juice containing 3% of the citric and malic acid extracts was regulated under 1×10^4 after 5 and 10 days of incubation (Fig. 5B). The 3% addition of the citric and malic acid extracts in the juice caused a little pH reduction from 3.6 to 3.5, and such pH difference did not affect the growth of the bacterium[21].

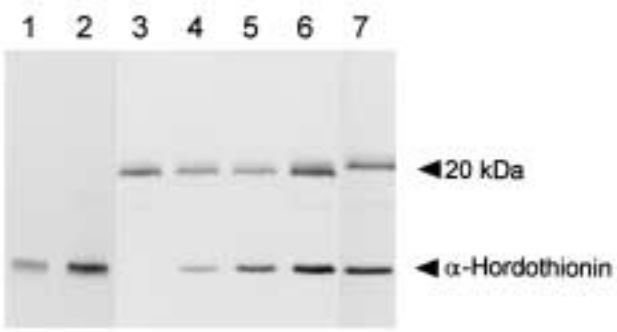

Photo.2 SDS-PAGE of HPLC fractions containing α-hordothionin. Lanes 1 and 2: standard α-hordothionin (1: 0.2 μg; 2: 0.5 μg); 3: water extract, 4-6: citric acid (4: 0.05 M; 5: 0.1 M; 6: 0.2 M) extracts; 7: 0.2 M malic acid extract.

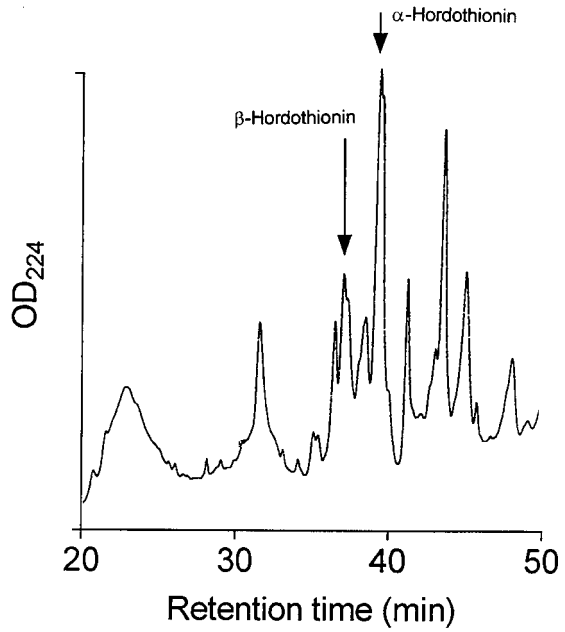

Fig.4 HPLC profile of 0.2 M citric acid extract from barley grains.

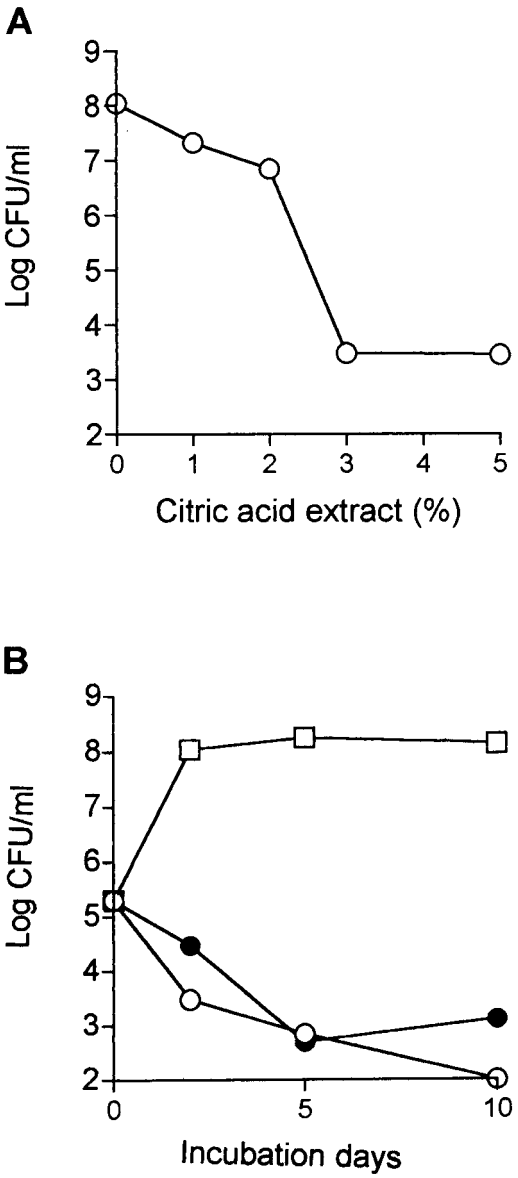

Fig.5 Growth inhibition of *A. acidoterrestris* by addition of barley extracts in satsuma mandarin juice. A: CFU after 2 days of incubation in the juice containing barley-0.2 M citric acid extract. B: open circles: 3% addition of the barley-0.2 M citric acid extract; closed circles: 3% addition of the barley-0.2 M malic acid extract; squares : no addition.

Ⅳ Discussion

The relation of *A. acidoterrestris* cell population and guaiacol accumulation in fruit juice has been reported[17),18)]. When the bacterium was grown at a rate exceeding 1×10^5/ml in fruit juice, the fruit juice contained a sufficient amount of guaiacol that had a noticeably tainted odor[18)]. Another report indicated that the guaiacol content in apple juice did not always correlate with the number of cells, whereas the minimum cell population for sensory taint was about 1×10^4/ml[17)]. Therefore, to keep the cell population of *A. acidoterrestris* under 1×10^4/ml prevents the spoilage of fruit juice, even if fruit juice is contaminated with some spores of *A. acidoterrestris*. Purified α-purothionin (20 μg/ml) regulated CFU in the satsuma mandarin juice and the mixed fruit-vegetable juice under 1×10^3/ml after 5 days of the incubation at 37℃ (Fig. 3). Although a slight difference in the average CFU was observed between the satsuma mandarin juice and the mixed fruit-vegetable juice, the cause is unknown. Also, 3% addition of the barley extracts with 0.2 M citric and malic acids regulated the average CFU in the satsuma mandarin juice under 1×10^3/ml after 5 days of the incubation (Fig. 5B).

In contrast, the inhibitory effect of α-purothionin against *A. acidoterrestris* was relatively weak in the 30% dilution of apple juice (Fig. 3). Apple juice contained a considerable amount of tannin[22)], which had ability to co-precipitate with various proteins[10)]. Therefore, α-purothionin added in apple juice may be co-precipitated with tannin and lose the antibacterial activity. Although many strains of *A. acidoterrestris* were grown in apple juice[9),17),18),21),26)], the strain of ATCC 49025 was not grown in 100% juice of the apple cultivar "Fuji" in this study. If many other strains of *A. acidoterrestris* are not grown in the apple juice, "Fuji" may be resistant to this bacterium.

Most fruit juice originally contains citric acid and/or malic acid, and the total concentration of these acids is around 1%. 0.2 M citric and malic acids correspond to 3.8% and 2.7% of the acids. The 3% addition of the 0.2 M citric or malic acid extract raises the concentration of citric acid (0.11%) or malic acid (0.08%) in fruit juice.

In Photo. 2, the silver-stained band of purified α-hordothionin (0.5 μg) was wide but pale compared

with the band of α-hordothionin in the HPLC fraction from the barly-0.2 M citric acid extract. If the amount of α-hordothionin in the HPLC fraction from the barly-0.2 M citric acid extract is maximally evaluated as 0.5 μg and the extract contains about a half amount of β-hordothionin (Fig. 3), the extract originally contains about 100 μg/ml of α-hordothionin and 50 μg/ml of β-hordothionin. Since the MIC of α- and β-hordothionins against *A. acidoterrestris* in the satsuma mandarin juice was 15 μg/ml, 10% addition of the barly-0.2 M citric acid extract seemed to be required for the control of the bacterium in the juice. However, *A. acidoterrestris* in the satsuma mandarin juice was actually controlled by 3% addition of the extract (Fig. 5). This result suggests that the barley extract contains antibacterial substances other than α- and β-hordothionins.

Barley seeds contain various antifungal proteins other than α- and β-hordothionins. A 30 kDa antifungal ribosome-inactivating protein (RIP) was extractable from barley seeds by 60 mM acetic acid[19]. RIP of an Andean crop *Milabilis expansa* inhibited the growth of a Gram-positive bacterium *Bacillus subtilis* [24], and the ribosomes of a Gram-negative bacterium *Escherichia coli* were inactivated by wheat leaf RIP, but not by wheat seed RIP[11]. Photo. 1 indicates that the barley extracts contained 30 kDa proteins. These reports and result suggest that the barley extracts using 0.2 M citric and malic acids may contain RIP, and the RIP possibly inhibit the growth of *A. acidoterrestris* in fruit juice. However, the heat-tolerance of barley RIP is unknown.

Antifungal radish 2S albumin and barley 2S albumin-like and Bowman-Birk inhibitors had synergistic antifungal effect with α-purothionin[23]. The radish 2S albumin alone inhibited the growth of a Gram-positive bacterium *Bacillus megaterium*, however, the antibacterial effect of the barley 2S albumin-like and Bowman-Birk trypsin inhibitors from barley has not been described[23]. Moreover, no synergistic antibacterial effect of the radish 2S albumin and α-purothionin was observed[23]. Barley seeds also contained antifungal proteins, i.e. chitinase[12),19)], β-glucanase[12)], thaumatin-like protein[8)], non specific lipid transfer protein[4)], and puroindoline[4)], but the antibacterial activity of these proteins alone has not been observed. However, synergistic antibacterial effect of α- and β-hordothionins and other antifunal proteins from barley is not yet examined.

Purified α- and β-purothionins were toxic to cultured mammalian cells[6)], but the oral administration of α- and β-purothionins to guinea pigs (103-229 mg/kg body weight) indicated no symptom within seven days[3)]. This may be because α- and β-purothionins are digested by trypsin and chymotrypsin[13)]. However, more study on safety of α- and β-thionins from barley and wheat is required. The citric and malic acid extracts of the barley grains contained considerable amounts of 12-16 kDa proteins (Photo. 1), which possibly include the trypsin/α-amylase inhibitor family[1)]. Since α- and β-thionins of barley and wheat are digestable by trypsin[13)], these thionins have not been reported to be allergenic for human. In contrast, the trypsin/α-amylase inhibitors are major allergens associate with baker's asthma[7),20)]. The inhibitors are not denatured by pasteurization because of heat- and acid-stabilities[2)].

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Summary

Heat-stable antimicrobial peptides, α- and β-hordothionins from barley and α-purothionin from wheat, inhibited the growth of thermoacidophilic spore-forming *Alicyclobacillus acidoterrestris* at a concentration of 5-10 μg/ml. Viable cells of *A. acidoterrestris* decreased in satsuma mandarin juice and mixed fruit-vegetable juice containing 20 μg/ml of α-purothionin, but the inhibition was relatively weak in 30% dilution of apple juice. α- and β-Hordothionins were extractable from barley seeds by 0.2 M citric and malic acids. The spoilage of satsuma mandarin juice by *A. acidoterrestris* was prevented by 3% addition of the barley extracts in the juice.

大麦・小麦α-およびβ-チオニンによる耐酸耐熱性細菌 *Alicyclobacillus acidoterrestris*の制御

老田　茂

摘　要

大麦および小麦由来で熱安定性が高い抗菌ペプチドのα-およびβ-チオニンは，耐酸耐熱性細菌である*Alicyclobacillus acidoterrestris*の増殖を5-10μg/mlで阻害した．20μg/mlの小麦α-チオニンを含むみかん果汁や果実・野菜ミックスジュース中でも*A. acidoterrestris*の生菌数が減少したが，30%希釈のリンゴ果汁中では阻害がやや弱かった．0.2 Mクエン酸およびリンゴ酸によって，大麦種子からα-およびβ-チオニンが抽出された．この大麦抽出液をみかん果汁へ3%添加したところ，*A. acidoterrestris*による果汁の腐敗が防止できた．