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Bitter Peptides Derived from α_{s1}-and β-Casein Digested with Alkaline Protease from *Bacillus subtilis*

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Abstract

The α_{s1}-and β-casein were purified by DEAE-cellulose chromatography and digested with alkaline protease from *Bacillus subtilis*. Bitter fractions from the hydrolyzates were isolated using n-butanol extraction, Sephadex G-25 gel chromatography, and high performance liquid chromatography. Peptide mixtures were separated by reverse-phase octadecyl silica column with linear gradient of 0-80% acetonitrile containing 0.1% trifluoroacetic acid. Major peaks were combined from replicate chromatographies and the bitterness of each peak was evaluated. The bitter-tasting peaks were rechromatograpied until isolated peaks were obtained. Three different bitter peptides(BP-I, BP-II, BP-III) were obtained from the α_{s1}-casein hydrolyzate. BP-I was eluted at 34% acetonitrile and BP-II, 35%, BP-III, 26%, respectively. Two bitter peptides(BP-IV, BP-V) were isolated from the β-casein hydrolyzate: BP-IV was eluted at 40% acetonitrile and BP-V, 42%. BP-V was the most hydrophobic peptide in the five bitter peptides. However, BP-I and BP-II tasted more bitter than BP-IV and BP-V.

Key words: bitter peptides, casein hydrolysate, alkaline protease

Introduction

Enzymic hydrolysis of proteins has been empolyed in food industry to improve functional properties such as solubility[1], to develop casein hydrolyzates for patients with digestive disorders, to minimize the allergy to milk proteins, and for the condition of cystic fibrosis. Enzymic hydrosis was preferred to acid hydrolysis because of the advantage of retaining the nutritional value of the proteins[2].

Of many proteases used in food industry for production of protein hydrolyzates, alkaline protease from *Bacillus subtilis* is the enzyme applied most often. Alkaline protease also can be used as an additive for the acceleration of cheese ripening[3]. In these applications, the occurrence of bitter flavor during the proteolysis is a major problem[4].

Bitterness has been attributed to the presence of bitter peptides which occur in fermented foods as well as in protease-treated proteins. A number of bitter peptides have been isolated and identified especially from various enzyme hydrolyzates of proteins and cheeses during the last two decades.

Matoba et al.[5] isolated a bitter peptide from tryptic hydrolyzate of casein. This peptide was the fragment 203-208 of β-casein. Pelissier et al.[6] identified eight bitter peptides which were isolated from α_{s1}-and β-casein digested with chymosin. These were the fragment 21-23, 29-32, 92-99, 99-101, 143-149, 145-151, 167-179 of α_{s1}-casein, and the fragment 103-105, 190-192, 203-209 of β-casein. Hill *et al.*[7] isolated four bitter peptides from hydrolyzed casein coprecipitate, which were identified as the fragment 23-24, 26-33, 91-100, 145-151 of α_{s1}casein. The major bitter-tasting peptide in papain-treated casein was the fragment 53-79 of β-casein[8]. Visser et al.[9] isolated three bitter peptides from rennet-treated casein, which were the fragment 128-139, 193-207, 193-209 of β-casein. The bitter peptides isolated from cheeses were the fragment 198-199 of α_{s1}-casein[10] and the fragment 46-67[11], 84-89[12], 193-207[12], 193-209[12] of β-casein.

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Opinions on the size of bitter peptides vary but it is generally agreed that they contain high proportion of hydrophobic amino acids such as leucine, phenylalanine, and proline[13]. Ney proposed that peptides with average hydrophobicity(Q-value) grester than 1400 cal/res taste bitter, while peptides with Q-value less than 1300 cal/res are not bitter, where Q is calculated from transition free energy of an amino acid from aqueous solution to an organic solvent[14]. So far, most of all bitter peptides isolated comply well with this "Ney's rule". The hydrophobicity of the peptide is the main factor determining the bitterness, but there seems to be other factors affecting the bitterness potency. The sequence and a spatial structure could be other factors, but these factors have not been fully studied[15,16].

In the present study, several bitter peptides were isolated from α_{s1}-and β-casein treated with alkaline protease from *Bacillus subtilis*, by solvent extraction, gel chromatography and high performance liquid chromatography to elucidate the mode of proteolysis by the enzyme, and to provide a basic information to minimize the bitter flavor of hydrolyzed proteins.

Materials and Methods

Alkaline protease from *Bacillus subtilis*

The alkaline protease which was known to produce bitter flavor from casein[20] was purchased from Sigma Co.(St. Louis, MO, USA). The specific activity of the enzyme was about 2,000U/mg according to the casein digestion method[17].

Preparation of α_{s1}-and β-casein

Whole casein was prepared from raw milk by double acid precipitation method[18]. The α_{s1}-and β-casein were isolated from whole casein by DEAE-cellulose column chromatography following the producere of Davies *et al.*[19]. Whole casein(1g) was alkylated with iodoacetamide and applied to a column(4×22cm) of DEAE-cellulose equilibrated with tris-Cl-urea butter(0.005M Tris, 0.03M NaCl, 6.0M urea, pH 8.60). Elution was with the same butter(300m*l*) followed by a linear gradient of NaCl in tris-Cl-urea butter(0.06M-0.25M). Chromatography was performed at 20℃ and fractions of 15m*l* were collected. Protein peaks were identified by electrophoresis.

Enzymic digestion of α_{s1}-and β-casein

Five hundred miligrams of α_{s1}-or β-casein were dispersed in 25m*l* of distilled water. The solution was adjusted to pH 9.0 by slow addition of 1N NaOH and subsequently heated at 66℃ for 20 min. For the formation of bitter peptides, 0.5mg of alkaline protease dissolved in 1m*l* of distilled water was added to the substrate solution, and the mixture was incubated at 40℃ for 20 hr, maintaining the pH at 9.0 by occasionally adding 1N NaOH. One drop of toluene was added to prevent bacterial contamination during the digestion.

Solvent extraction of bitter components

Hydrophobic components of the α_{s1}- or β-casein hydrolyzate were extracted with butanol. Immediately after 20hr digestion, equall volume of butanol was added to the protein hydrolyzate and this mixture was vigorously stirred for 2 hr. The butanol phase was separated from the water layer using a separatory funnel after setting overnight at 4℃.

Gel chromatography of bitter extract

The bitter extract was dissolved in 10m*l* of distilled water at pH 7.0 and insoluble materials were removed with Whatman membrane filter(pore size; 0.45μm). This filtrate was applied to the Sephadex G-25 column(2.5×90cm) and eluted with distilled water at a flow rate of 20m*l*/hr. Absorbance at 230nm was measured for each of 5m*l* fractions. Fraction tubes that form each peak at the chromatogram were pooled, freeze-dried, and evaluated for the bitterness.

High performance liquid chromatography

Separation of bitter peptides from bitter fraction from gel chromatography was done using HPLC system(Waters Assoc.) eqipped with reversephase, octadecyl silica column (μBondapack C_{18}, 10μm, 30cm×3.9mm, 10% carbon). The liquid chromatograph was composed of two pumps(model 6000A pump and model 45 pump), model 720 system controller and a model U6K injector, coupled to a model 450 variable-wavelength UV detector and a model 730 data module.

A 0.1% trifluoroacetic acid(TFA)/80% acetonitrile system either in a stepwise or linear gradient was empolyed as the eluent, and the detector monitored the absorbance at 230nm. All the elutions were done at a flow rate of 1.0ml/min at the ambient temperature. The chromatograms were divided into several fractions and each fraction was collected and pooled by repeated injections. The bitter-tasting fractions were rechromatographed until isolated peaks were obtained.

Sensory evaluation of bitterness

Evaluation of the bitter taste was done at each stage of enzymic hydrolysis, solvent extraction, gel chromatography, HPLC, and rechromatography of HPLC. Sensory evaluation was done by selected panels of 1-2 persons. Samples(50-100μl) of 0.1% aqueous solution of the peptides were administered directly onto the rear taste buds that are most sensitive to bitter taste. The bitterness of the sample was marked as one of 0 (not detectable), +(very slight), ++(slight), +++(definite), ++++(pronounced).

Results and Discussion

Fig. 1 shows the chromatogram of DEAE-cellulose column chromatography of alkylated whole casein. Major casein fractions, i.e., α_{s0}, α_{s1}, α_{s2}, β, and κ-caseins, were identified by the method of Davis and Law[19]. To obtain more purified

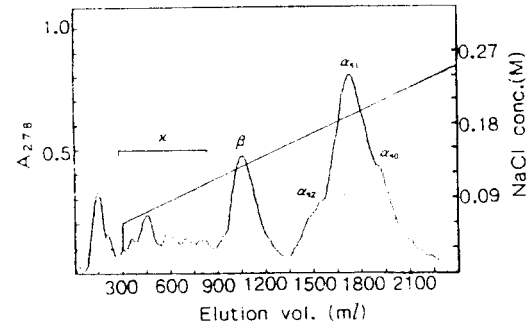

Fig. 1 The fractionation of alkylated whole casein by DEAE-cellulose column chromatography

α_{s1}-casein, the shoulders in the α_{s1}-casein peak were discarded.

When α_{s1}-and β-casein were digested with alkaline protease, bitter taste appeared with the progress of hydrolysis and reached a maximum after 20hr digestion. Prolonged digestion over 20hr did not increase the bitterness. After the extraction with n-butanol, bitter taste was predominant in the butanol phase. The interface between water and butanol phase showed slight bitterness and the flocculent materials were concentrated in this phase.

Sephadex G-25 gel chromatogram of bitter extract from α_{s1}-casein hydrolyzate is shown in Fig. 2. This chromatogram was divided into four fractions(α-I, α-II, α-III, α-IV), each pooled fraction was freeze-dried, and evaluated for the bitterness. The α-II fraction was tasted as definite bitter(+++), and α-III fraction, pronounced bitter(++++).

Fig. 3 shows the Sephadex G-25 gel chromatogram of bitter extract from β-casein hydrolyzate. Five peak fractions(β-I, β-II, β-III, β-IV, β-V) were tested for bitterness, and the β-III fraction showed definite bitterness(+++). The β-II and β-IV fraction were tasted as slightly bitter but probably because of the contaminated β-III fraction.

Fig. 4A is the HPLC chromatogram of the bitter fraction α-II in the Fig. 2. The freeze-dried α-II

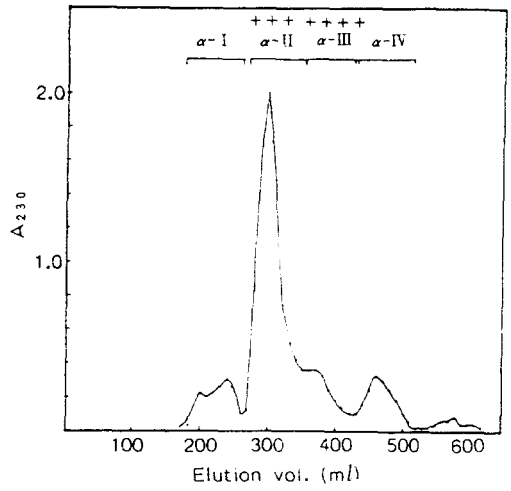

Fig. 2. Sephadex G-25 gel chromatogram of bitter extract from α_{s1}-casein hydrolyzate.

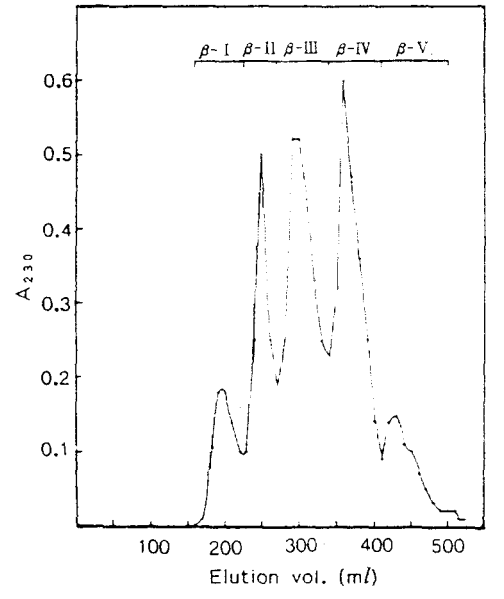

Fig. 3. Sephadex G-25 gel chromatogram of bitter extract from β-casein hydrolyzate.

fraction was analyzed by HPLC at a linear gradient of 0.1% TFA/80% acetonitrile(buffer B) over 80min. Of more than 25 peaks in the figure, four fractions(RT 26.88, RT 28.70, RT 32.51, RT 36.21) at later part of the chromatogram(more hydrophobic) were collected manually. This HPLC chromatography was repeated several times and the same peaks(at the same retention time) were pooled, freeze-dried, and sensory-analyzed. One peak(RT 36.21) showed definite bitter taste, and was subjected to HPLC rechromatography.

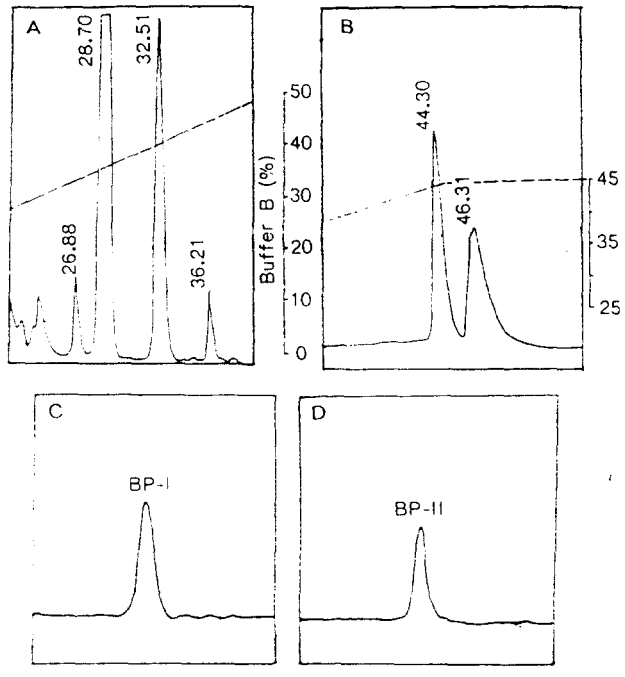

Fig. 4. HPLC chromatograms of bitter peptides from fraction α II in Fig. 2.
A: fraction α II B: RT 36.21 peak in A
C: RT 44.30 peak in B D: RT 46.31 peak in B

Fig. 4B shows HPLC chromatogram after the rechromatography of the bitter-tasting peak(RT 36.21) in Fig. 4A. In this rechromatography, the gradient profile was such programmed that the peak comes out at a constant composition of the eluent. In isocratic systems, the contaminated peak that was not resolved in gradient systems could be resolved. Two peaks appeared, of which retention times were 44.30 and 46.31, respectively. These two peak were collected separately and pooled from several repeated chromatographies for sensory analysis. Both peaks tasted bitter

equally and further rechromatographed.

The final rechromatograms containing only one bitter peptide peak were shown in the Fig. 4C and 4D, respectively. The RT 44.30 peak in Fig. 4B was eluted at 42% buffer B(80% acetonitrile) and named BP- I (Fig. 4C), and the RT 46.31 peak in Fig. 4B was eluted at 43% buffer B(35% acetonitrile) and named BP-II (Fig. 4D).

The HPLC chromatogram of the bitter fraction α-III in Fig. 2 is shown in Fig. 5A. Under the same condition as in Fig. 4, four fractions(RT 25.00, RT 27.50, RT 30.69, RT 32.15) were collected, pooled, and sensory-analyzed. All four fractions tasted bitter but not so strong. It was thought that each of these four fractions contributed to the total bitterness of α_{s1}-casein hydrolyzate, but the fraction of RT 30.69 tasted more bitter than other three. Fig. 5B shows the final rechromatogram of the purified BP-III of which retention time was 32.09 min(26% acetonitrile).

Fig. 6A shows the HPLC chromatogram of the bitter fraction β-III in the gel chromatogram of Fig. 3. A linear gradient of 0.1% TFA/80% acetonitrile(buffer B) over 100min was applied. Four peaks eluted at later part of elution(RT 45.80, RT 49.77, RT 51.50, and RT 55.27) were collected manually. This chromatography was repeated several times and the same peaks at the same retention time were pooled, freeze-dried, and sensory-analyzed. The peak of which retention time 49.77 was tasted slightly bitter(+ +). The peak of RT 51.50 tasted definitely bitter(+ + +), and was subjected to HPLC rechromatography(Fig. 6B).

In the Fig. 6B, 0-50% buffer B over 10 min was delivered, and 50-60% buffer B, over the next 10 min. Two major peaks were collected and pooled from repeated chromatographies, freeze-dried, and sensory-analyzed. Both peaks were slightly bitter. These two peaks were subjected to further rechromatographies.

Fig. 6C and Fig. 6D shows the final HPLC chromatograms of above two bitter-tasting peaks. The BP-IV in Fig. 6C was eluted isocratically at 50% of buffer B. In Fig. 6D, the isolated BP-V was eluted at 53% buffer B(42% acetonitrile).

In the five bitter peptides isolated, BP-V originated from β-casein was most hydrophobic. However, BP- I and BP-II tasted more bitter than BP-IV and BP-V.

The primary structure of these five bitter peptides should be analyzed by means of amino acid, end-group, and sequence analysis to identify the fragment of the respective casein molecule. Then the relationship between bitter flavor and average

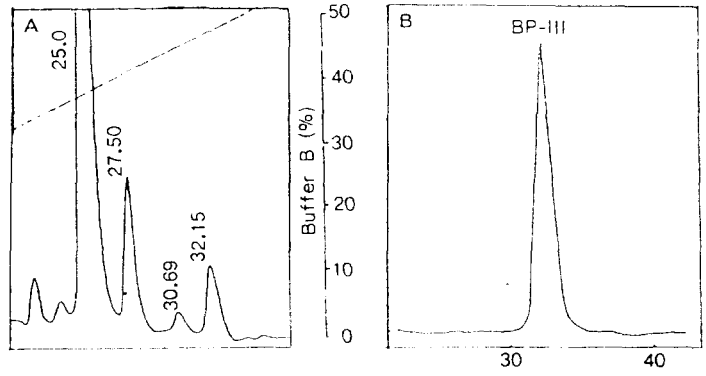

Fig. 5. HPLC chromatograms of bitter peptide from fraction α III in Fig. 2.
A: fraction α III in Fig. 2 B: RT 30.69 peak in A

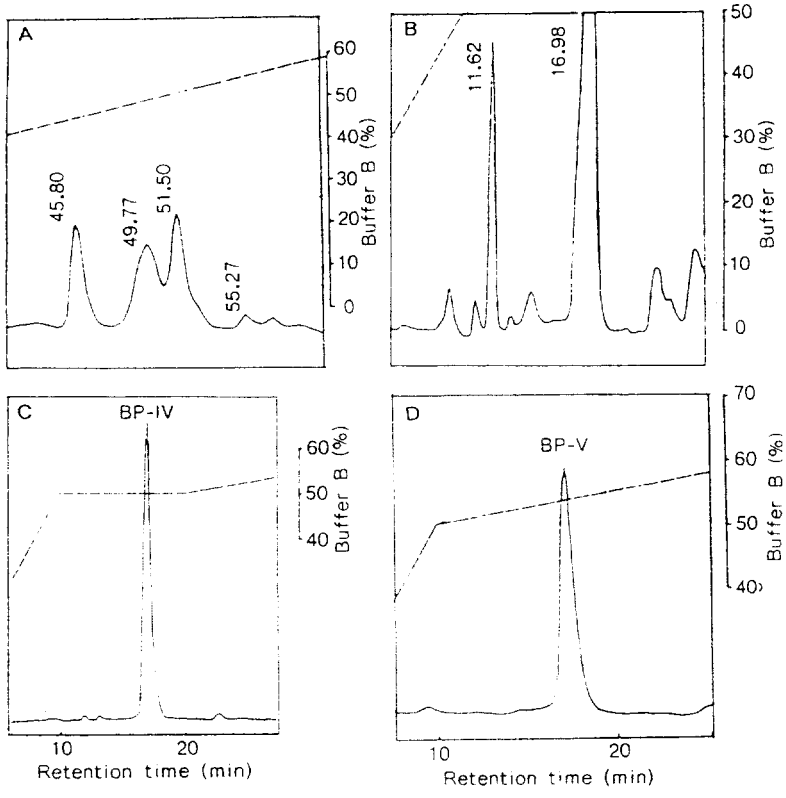

Fig. 6. HPLC chromatograms of bitter peptides from fraction β III in Fig. 3
A: fraction β III B: RT 51.50 peak in A
C: RT 11.62 peak in B D: RT 16.98 peak in B

hydrophobicity of the peptides also could be discussed.

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*Bacillus subtilis*의 염기성 프로테아제로 분해된 α_{s1}- 및 β-카세인에서 분리된 쓴 맛 펩타이드

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식품에서의 쓴 맛 펩타이드의 형성 기작 및 분해에 의한 제거 방법을 알아보기 위하여 카제인 가수 분해물에서 쓴 맛 펩타이드를 분리하여 그 특성을 조사하였다. *Bacillus subtilis*가 생산하는 염기성 단백분해 효소를 순수 분리된 α_{s1}-카제인과 β-카제인에 처리하여 카제인 가수 분해물을 만들고, 이 가수 분해물에서 용매 추출, Sephadex G-25 겔 크로마토그래피, 고성능 액체 크로마토그래피를 이용해 쓴 맛 펩타이드를 순수 분리했다. 고성능 액체 크로마토그래피는 역상 칼럼인 octadecyl silica 칼럼을 사용했고, 0.1% TFA와 80% CH_3CN의 linear gradient 방법을 이용했다. α_{s1}-카제인 가수 분해물에서 3가지 쓴 맛 펩타이드를 분리하였는데, BP-I은 CH_3CN 34%에서, BP-II는 35%, BP-III는 26%에서 각각 용출되었다. β-카제인 가수 분해물에서는 2가지 쓴 맛 펩타이들 분리했는데, BP-IV는 CH_3CN 40%에서, BP-V는 42%에서 용출되었다. 분리된 다섯 가지 펩타이드 중 BP-V가 가장 소수도가 높았으며, 쓴 맛은 BP-I과 BP-II가 가장 강했다.