Studies on antihypertensive peptides derived from whey Protein(乳精たんぱく質から誘導される降血圧ペプチドに関する研究)

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論 文 内 容 要 旨

STUDIES ON ANTIHYPERTENSIVE PEPTIDES DERIVED FROM WHEY PROTEIN

INTRODUCTION

A number of functional peptides relating to antihypertensive effects derived from bovine casein (3, 4), human casein (1) and whey protein (WP) (2, 5, 7), have already been identified and characterized.

ACE (EC 3.4.15.1) is classified as a peptidyl-dipeptide hydrolase liberating exopeptidase which has been classically associated with the renin angiotensin system regulating peripheral blood pressure. The renin angiotensin system has been implicated in blood pressure regulation and hypertension. Renin acts on angiotensinogen and releases inactive angiotensin I, which is then converted to the active peptide hormone angiotensin II by ACE (6).

Application of enzymatic hydrolysis to improve the functional properties of WP were described. Bioactive peptides from WP and their physiological effects have received less attention than those from casein (2). However, limited studies have been carried out on WP derived angiotensin converting enzyme (ACE) inhibitory peptides. Nevertheless, some of the antihypertensive

effect related to ACE inhibitory peptides was derived from WP such as lactorphins which are involved in the sequences of bovine α-Lactalbumin (α-La)/or β-Lactoglobulin (β-Lg) (5) and albutensin A derived from bovine serum albumin (BSA) (7).

CHAPTER 1

Part 1: New Derivation of The Inhibitory Activity against Angiotensin Converting Enzyme from Caseinoglycopeptide (CGP, κ-Cn: f 106 - 169).

One of component of sweet cheese whey which liberated from κ-casein by chymosin digestion during the cheese making process is CGP. CGP is expected to be one of the precursor of bioactive peptides that are inactive while they are included within the sequence of the precursor protein and may become as bioactive digestion products. In this part, the induction of ACE inhibitory peptides from CGP by hydrolysis with proteases was tasted, and isolate of the active component were performed.

After 24 h digestion of CGP by 5 different proteases, the strongest ACE inhibitory activity (92.6%) was derived by the pepsin digest. The activity was dependent on the digestion time, and that of 24 h digestion was higher than 12

h (21.9%) or 48 h (35.7%). The pepsin digest of CGP was first fractionated by C-4 hydrophobic chromatography, and then by C-18 hydrophobic chromatography.

One of an ACE inhibitory peptides was isolated by hydrophobic and reversed-phase high performance liquid chromatography (RP-HPLC). The amino acid sequence analysis of the peptide was clarified as NH_2-Ile-Ala-Ser-Gly-Glu-Pro-COOH (κ-Cn: f125-130) and was named "κ-caseinosin".

Part 2 : New Derivation of The Inhibitory Activity against Angiotensin Converting Enzyme from Whey Protein.

The most potent ACE inhibitory activity (91.91%) was derived from whey protein (WP) containing CGP by papain digestion. WP removed CGP and cheese whey powder (CWP) showed the highest activity after thermolysin digestion of 95.23% and 98.56%, respectively. No significant differences in inhibitory activity were observed between WP with and removed CGP samples. ACE inhibitory peptides are considered to come mainly not from CGP but from other WP components such as β-Lg, α-La, BSA, proteose-peptone. Furthermore, a similar experiment with WP containing CGP and CWP concluded that lactose and minerals in CWP do not contribute to the ACE inhibitory activity.

CHAPTER 2

Identification of An Antihypertensive Peptide Derived from Whey Protein by Protease Digestion.

The digested WP by 7 kinds of proteases were assayed for the ACE inhibitory activity and for the systolic blood pressure (SBP) effect of spontaneously hypertensive rats (SHR) after gastric intubation. Digestion of WP by proteinase K gave strong depressive effect on the SBP(−55 mmHg), after 6 h after gastric intubation.

Six peptides were chromatographically isolated from the proteinase K digest by a combination of HPLC with RP and gel filtration modes. The amino acid sequences and the origins of 6 peptides were clarified as follows : Val-Tyr-Pro-Phe-Pro-Gly [β- Casein (Cn): f 59–64]; Gly-Lys-Pro (β_2-microglobulin: f 18–20); Ile-Pro-Ala (β-lg: f 78–80); Phe-Pro (BSA: f 221–222; β-Cn: f 62–63, f 157–158, and f 205–206); Val-Tyr-Pro (β-Cn: f 59–61) and Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro (β-Cn: f 80–90). Chemically synthesized peptides, except an undecapeptide (β-Cn: f80-90), showed the antihypertensive activity in SHR. The synthetic tripeptide (Ile-Pro-Ala), showed the strongest antihypertensive activity (−31 mm Hg).

CHAPTER 3

Detection of Antihypertensive Tripeptide Ile-Pro-Ala (β-lactosin A) in Lung of SHR after Gastric Intubation.

Detection and isolation of tripeptide Ile-Pro-Ala (IPA) after gastric intubation was studied in organs (lung, kidney, heart and testes) of SHR. ACE activity in lung, kidney and testes of SHR given standard tripeptide IPA were lower than those ACE activity of SHR given distilled water. Only a peak with the same retention time (Rt) of IPA was detected from the lung of SHR by HPLC analysis. No peak with the same Rt of IPA was detected from kidney, heart and testes of SHR or in rats given distilled water. By analysis of amino acid composition, amino acid sequence and molecular mass, the peptide isolated from lung of SHR was confirmed to be IPA. These results indicated that administrated IPA is absorbed directly from intestine within 6 hours after gastric intubation without being decomposed by digestive enzymes, then shows the antihypertensive effects in SHR by inhibiting the ACE.

CHAPTER 4

Application Trial of The Development of A New Type of Fermented Cheese Whey Beverage with Antihypertensive Effects.

Part 1: Laboratory condition

Strong ACE inhibitory activity more than 95% was derived by proteinase K and thermolysin digestion of CWP under experimental conditions in laboratory. The digested CWP was fermented at 37°C for 24 hr with 2% (v/v) inoculation of 2 kinds of lactic acid bacterial culture (each 1% of *L. delbrueckii* ssp. *bulgaricus* NIAI B6 and *St. thermophilus* NIAI 510). Through the comparison of the ACE inhibitory activity before and after lactic acid fermentation, proteinase K was selected as the most prominent enzyme among the 7 proteases tested, because almost no decrease in activity after fermentation (from 89.9 to 89.8%) was observed.

The analysis of viable cells in the sample after fermentation indicated that enough viable cells (2.5×10^8/ml) could remain in the beverage.

Part 2: Industrial condition

CWP was digested with four kinds of food-additive proteases (protease A, actinase As, kokulase P and denatyme AP) under industrial conditions at 37°C for 8 h. The digested samples were assayed for ACE inhibitory activity and

were evaluated on the changes in SBP of SHR after gastric intubation. Although ACE inhibitory activity of actinase As digest of CWP was weak (44.1%), a strong decreasing effect of SBP (-32 ± 9.54 mm Hg) was observed. The actinase As digest of CWP was followed by fermentation by two kinds of lactic acid bacteria (the same strain used in part 1) at 37°C for 8 h. Peptides were fractionated by hydrophobic chromatography using Wakogel LP-40C18 resin with stepwise elution from 15% to 90% (v/v) using CH_3OH. A fraction eluted with 45% CH_3OH showed the highest antihypertensive activity (-21 x 3.61 mm Hg) against SHR.

Finally, two peptides were chromatographically isolated from actinase As digest by a combination of HPLC with RP and gel filtration modes. The amino acid sequences and the origins of 2 peptides were clarified as follows: Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln (tridecapeptide, α_{s1}-Cn: f 1-12) and Val-Arg-Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys-Phe-Asp-Lys-Ala (heptadecapeptide, β-Lg: f 123-139). Chemically synthesized tridecapeptide showed the antihypertensive activity (-18 ± 4.57 mmHg) in SHR and the IC_{50} was 278 μM.

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Original papers:

1 Amhar, A., T. Saito., M.V. Aimar and T. Itoh. 1996. New derivation of the inhibitory activity against angiotensin converting enzyme (ACE) from sweet cheese whey. *Tohoku J. Agric. Res.* **47**: 1-8.

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Presentation in International Conference.

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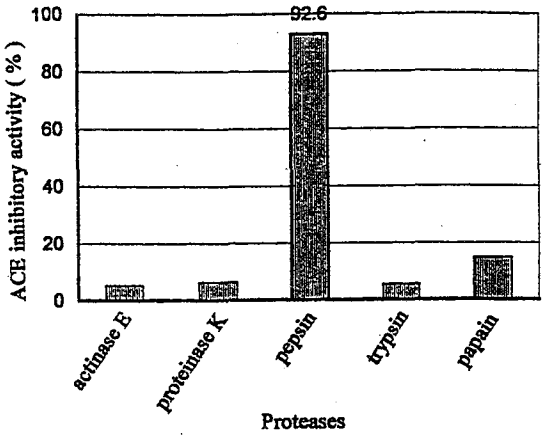

Fig. 1 ACE inhibitory activities derived from caseinoglycopeptide (CGP, κ-Cn: f106-169) by digestion with 5 kinds of proteases

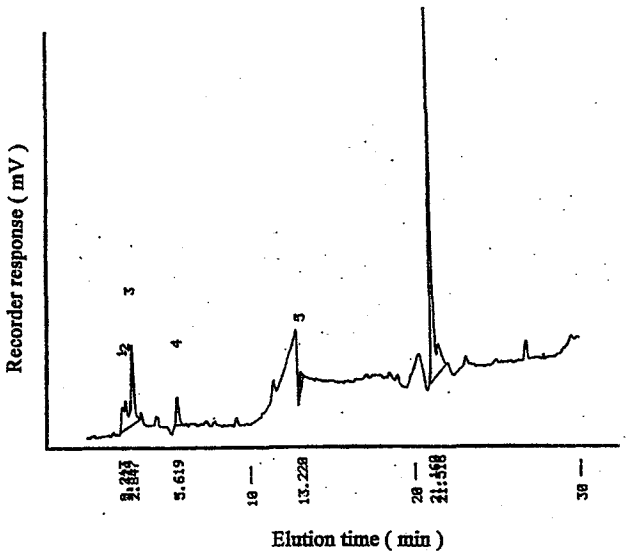

Fig. 2 An elution profile of the purified peptide from Fig.1 (digested CGP by pepsin) by reversed phase HPLC analysis

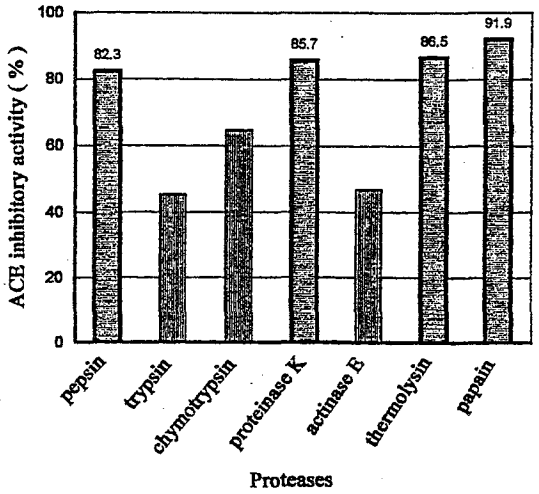

Fig. 3 ACE inhibitory activity derived by hydrolysis of whey protein (WP) sample containing CGP with 7 proteases

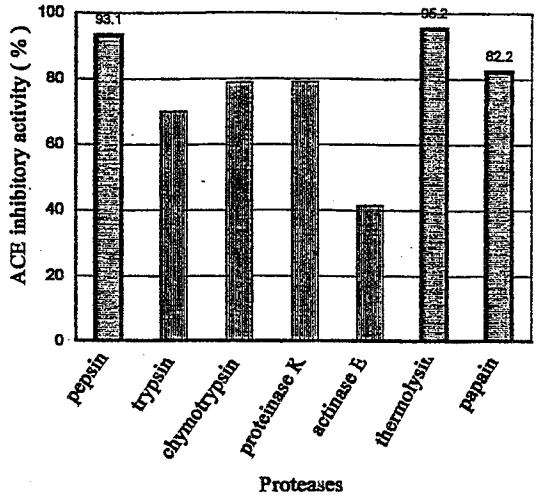

Fig. 4 ACE inhibitory activity derived by hydrolysis of WP removed CGP with 7 proteases

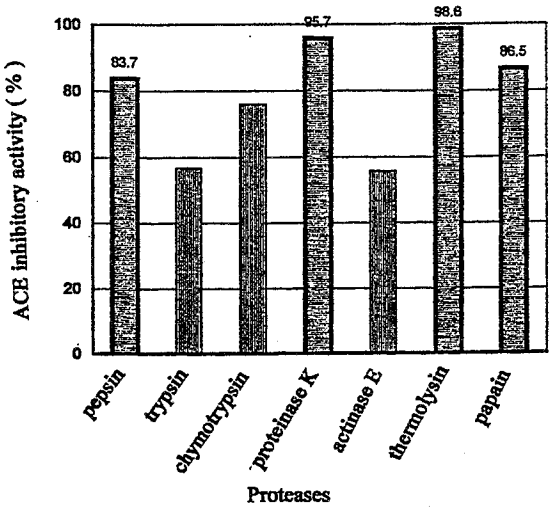

Fig. 5 ACE inhibitory activity derived by hydrolysis of cheese whey powder (CWP) with 7 proteases

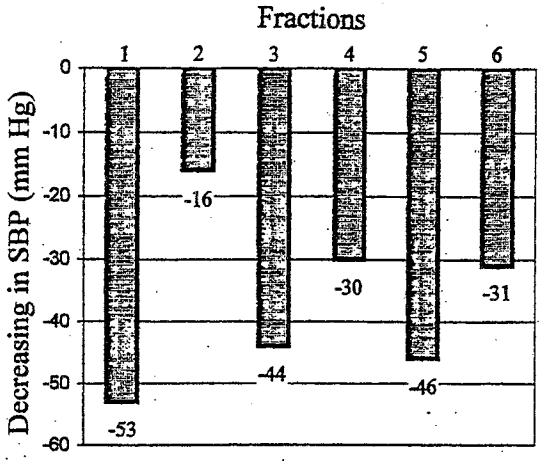

Fig. 6 Antihypertensive effects to SHR of WP digest with proteinase K fractionated by hydrophobic chromatography. Fraction 1:40% methanol eluant. Fraction 2: 40-30%; 3:40-33%; 4:40-36%; 5:40-39% and 6:40-42% methanol eluant. Dose 8 mg/kg, changes on SBP in SHR at 6 h after a gastric intubation of the sample

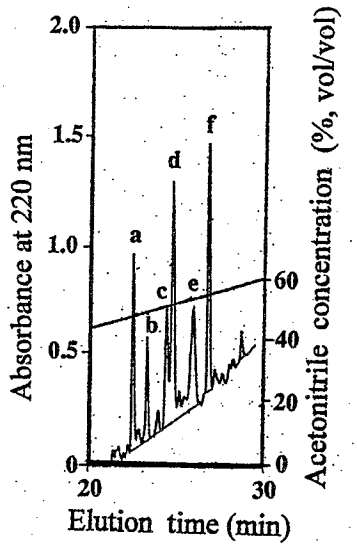

Fig. 7 An elution profile of the fraction 5 from Fig. 6 by RP-HPLC analysis

Table 1 The antihypertensive and the ACE inhibitory activity of synthetic peptides

Sample	Sequence	Origin	SBP [1]		Molecular mass	IC_{50} [2]
			—(mm Hg)—		$(M+H)^+$	(μM)
			X	SE		
a	VYPFPG	β-CN[3]: f59-64	−22*	4.6	678.86	221
b1	GKP	β_2-m: f18-20	−26*	4.4	300.39	352
b2	IPA	β-LG: f78-80	−31**	6.1	299.39	141
c2	FP	BSA: f221-222	−27*	4.4	262.32	315
		β-CN[3]: f62-63				
		β-CN[3]: f157-158				
		β-CN[3]: f205-206				
d	VYP	β-CN[3]: f59-61	−21*	4.6	377.47	288
f	TPVVVPPFLQP	β-CN[3]: f80-90	−8	2.6	1193.6	749
κ-caseinosin	IASGEP	κ-CN: f 125-130	−22.5	8.3	554.6	2859.4

[1]Systolic blood pressure. Changes on SBP in SHR at 6 h after a gastric intubation. Mean of three determinations.
[2]The concentration of peptide needed to inhibit 50% of the ACE activity.
[3]existed as proteose −peptone (β-casein fragment hydrolyzed by plasmin) in cheese whey.
Dose of 8 mg/kg. β_2-m = β_2-Microglobulin. *Significant difference from the control ($P<0.05$). **Significant difference from the control ($P<0.01$).

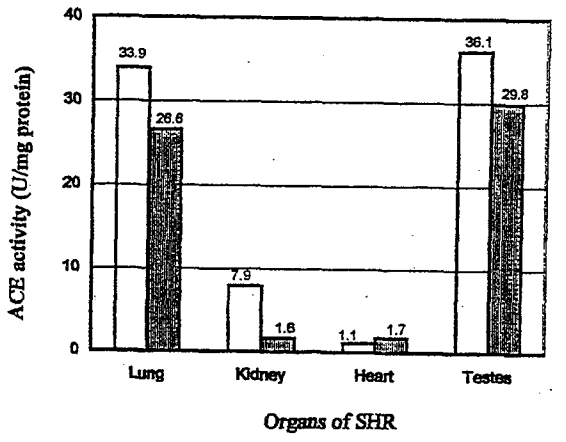

Fig. 8 The ACE activity in lung, kidney, heart and testes of SHR at 6 h after administration of distilled water (d.w.) or chemically *synthesize* IPA. White bar: d.w.; black bar: IPA

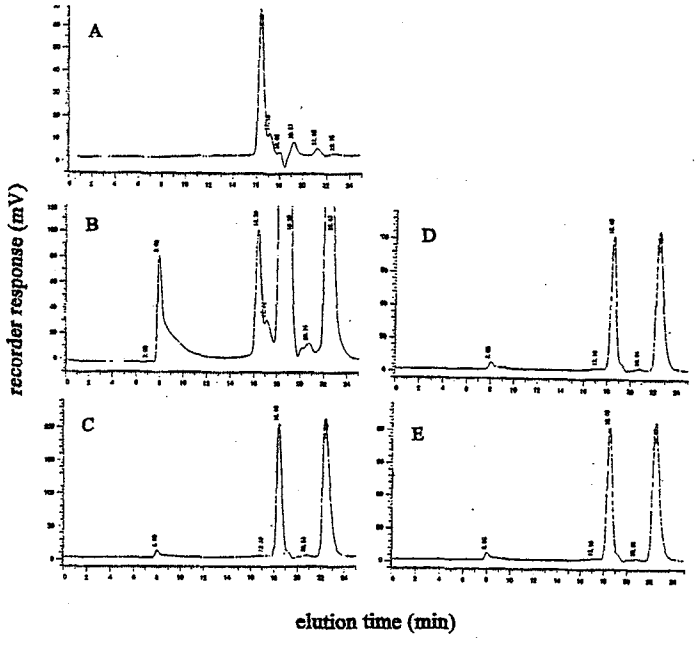

Fig. 9 Elution profiles by gel filtration HPLC of the standard IPA (A); Isolated peptide from lung (B); kidney (C); heart (C) and testes (E).

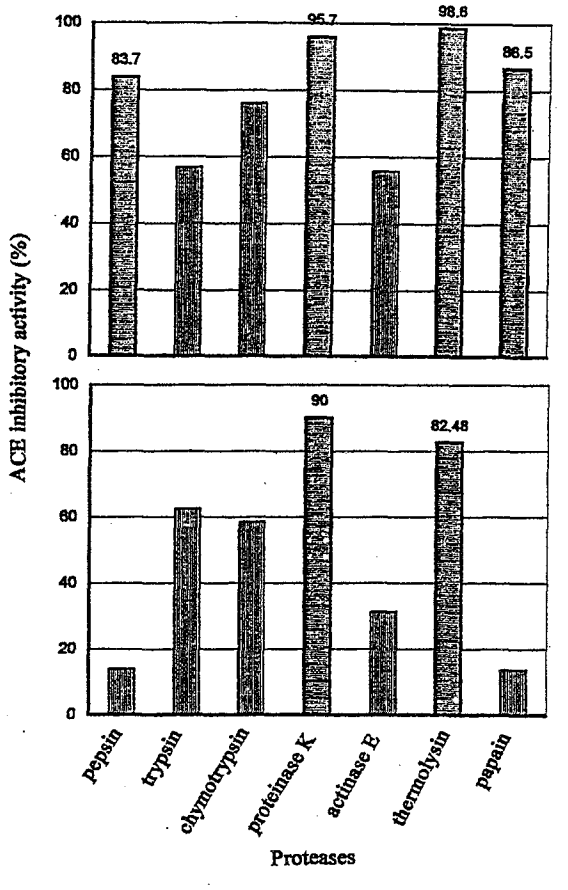

Fig. 10 The comparison of ACE inhibitory activity derived from CWP by protease digestion in optimum buffer (A) or in distilled water (B)

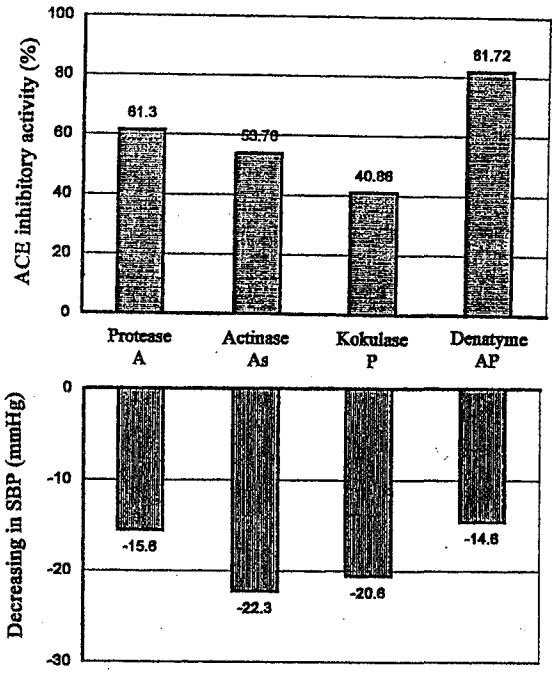

Fig. 11 ACE inhibitory activity (A) and antihypertensive activity (B) derived from CWP which were digested by 4 kinds of proteases after fermentation with lactic acid bacteria

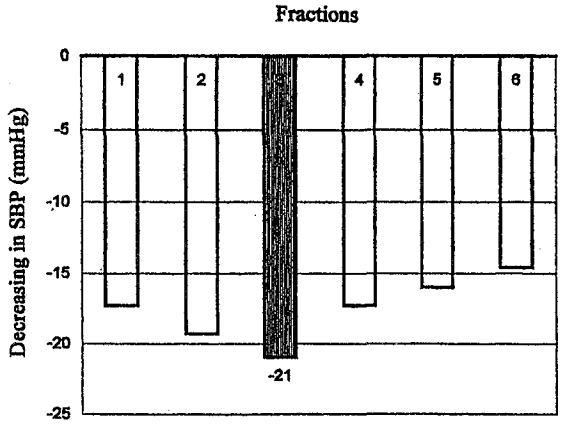

Fig. 12 Antihypertensive effects of 6 fraction of WP digested with actinase As and fermented by 2 kinds of lactic acid bacteria, fractionated by hydrophobic chromatography. Fraction 1: 15%; 2:30%; 3:45%; 4:60%; 5:75% and 6:90% of methanol eluant

Table 2. Amino acid sequences, molecular weights by tof mass analysis, and their origin of antihypertensive peptides derived from fraction 3 in Fig. 12.

Peptides	Sequence[1]	Mass[2] (M+H)+	Original
a	RPKHPIKHQGLPQ	1541.7	αS1-Cn (f1-12)
e	VRTPEVDDEALEKFDKA	1971.5	β-lG (f123-139)

[1]Amino acid sequences were analyzed using a protein sequencer.
[2]Molecular weights were analyzed by maldi tof mass analysis.

論文審査結果要旨

牛乳のホエーは，チーズを生産する際の副産物として生成するものであり，原料乳の90%がホエーとなるため，その利用法の開発が世界的に問題となっている。本研究は，ホエータンパク質をプロテアーゼで処理することによって降血圧ペプチドを生成しておき，これをさらに乳酸菌で発酵することによって血圧降下作用のある発酵ホエードリンクを作ることを目指したものである。

タンパク質のプロテアーゼ分解によって生成するペプチドの中には，アンジオテンシン変換酵素(ACE)を阻害することによって血圧の上昇を抑制する作用のあるものが多く見出されている。牛乳タンパク質の中では，カゼイン由来のペプチドから多く見出されているがホエータンパク質からは少ない。

本研究ではまず，チーズホエー中にκ-カゼインの断片であるカゼイノグリコペプチドが含まれていることから，この酵素分解による降圧ペプチドの生成について調べた。ペプシン消化物中に強いACE阻害活性が認められ，これを分画し，構造解析することによって，6アミノ酸から成るペプチドを見出し，κ-カゼイノシンと命名した。次いで，全ホエータンパク質を7種のプロテアーゼで消化し，ACE阻害活性と高血圧自然発症ラット(SHR)を用いて降圧効果を測定したところ，プロティナーゼKが最も効果が高かったことから，この消化物からペプチドを分画精製することによって，6種のペプチドを単離し，その構造を解明した。プロティナーゼ K で処理したチーズホエーを，更に*L.bulgaricus*と*Str.thrmophilus*の2種の乳酸菌で発酵させ，乳酸菌数および発酵前後の降圧効果を調べた結果，充分な数の乳酸菌を含む，降圧作用を保ったホエードリンクの作成できることが認められた。

次に，経口投与した降圧性ペプチドが，腸管から吸収され，器官に到達して効果を発揮しているかどうかを確かめるため，降圧効果の高かったトリペプチドIle-Pro-Ala(IPA)を合成し，ラットに経口投与したのち，肺，腎臓，心臓，精巣を摘出しそれぞれからIPAの回収を試みた。その結果肺からIPAが回収され，腸から吸収されたIPAが肺に到達し，ACEと結合することによって効果を現していることを確認した。

降圧ペプチドを含む発酵ホエードリンクを商品として仕上げるため，食品添加物として認可されている5種のプロテアーゼを用いてホエーを処理したところ，アクチナーゼAsによる処理で最も高い降圧効果が認められた。この中からは，13および17のアミノ酸から成る降圧性ペプチドが得られ，それらの化学構造を明らかにした。プロテアーゼ処理後，乳酸菌で発酵させることにより，降圧作用を保った発酵ホエードリンクが得られた。

このようにして，チーズホエーより降圧作用を持つ発酵ホエードリンクを作るための，基本的な工程が定められ，ホエーの有効利用に道をひらくものとしての価値が高いと考えられた。よって審査員一同は，本研究者に博士(農学)の学位を授与するに値するものと認定した。