ペプチド装置の開発で進む有用ペプチドの機能解明

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食品タンパク質の酵素分解物の経口摂取により、血圧低下、食後の高脂血の改善等の有益な作用が報告されている。これらは医薬品と同様に活性分子と生体の相互作用によると考えられる。しかし、食品タンパク質の酵素分解物中には非常に多くの種類のペプチドが存在する。またほとんどのペプチドは消化・吸収の過程でアミノ酸に分解される。そのため、従来から行われてきた試験管内でのアッセイに基づき活性成分を同定することは非常に困難である。一方、経口摂取によるアッセイで活性成分を同定するためには、かなりの量のペプチドを分画する必要がある。しかし、ペプチドを有害な試薬を用いずに大量に分画することはかなり困難であった。

我々はこの問題を解決するため、ここのペプチドの等電点の差に注目し、ペプチド自身を両性担体として用いる等電点電気泳動法が有効であることを示し、これを Autofocusing と呼んでいる。動物実験のため最大 50 L までのサンプルが分画可能なバッチ型 Autofocusng 装置の開発に成功し、この装置を用いて in vivo のアッセイのみで鮫軟骨由来の尿酸低下ペプチド、グルテン由来の肝炎抑制ペプチドの同定に成功している。これらの結果に基づき、in vivo の評価に基づく、in vivo activity-guided fractionation を機能性ペプチドの同定法として提案している。

さらの連続分画が可能な Autofocusing 装置の開発にも成功し、食品加工への応用の可能性をしめしている。

食品成分の摂取により従来の栄養的な価値を超えた健康増進作用が示唆されている。

高血圧の緩和、メタボリックシンドロームの改善、高尿酸血症の緩和、皮膚・関節の状態改善、メンタル面の改善…

特に食品タンパク質の酵素分解物であるペプチドは、

•ヒトへの応用は粗分画物なら容易

•非常に複雑な多数の成分が存在

•ペプチド等は生体中でさらに分解する可能性が高く、vitroとvivoの評価が一致しない可能性が高い。

•Vivoでは混合物が評価に使われ、生体への移行がほとんど不明であり、活性成分は、ほとんど不明。

食品は基本的に食経験があるものを対象

ヒトでの評価のハードルが比較的低い

活性成分の同定が非常に困難

複雑な構造の成分でも利用可能

化学反応による活性の改変は困難

消化・吸収過程での変化が大きく、それを把握しにくい

そのため In vitro high throughput screening → Animal experiment →Human trialといった従来の発想ではvitroで活性があった成分がHuman trialで活性がなければ、すべての努力が無駄になる可能性がある。

そこで、機能性食品の開発、特にペプチド性の機能性食品のために、Vivoでの評価に基づく活性成分の同定→In vitroの実験でのメカニズム解明のアプローチを提案する。

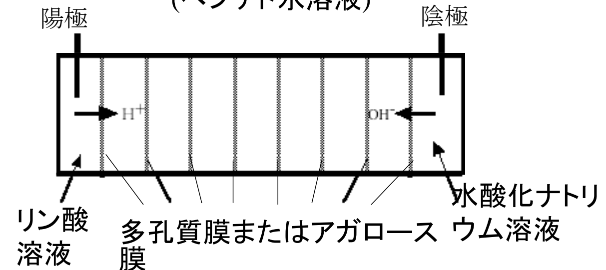
提案

•大量分画によるvivoで活性成分の同定

•標的組織に移行した活性成分の同定

上記の手法により決定した活性成分を用いてメカニズムの解明

サンプルコンパートメント
(ペプチド水溶液)



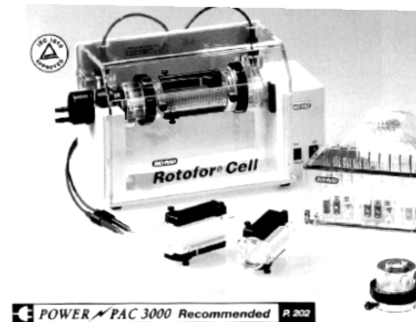
Autofocusingの原理　化学合成アンフォライン を加えることなくサンプルペプチドが両性担体として自らの等電点に移動

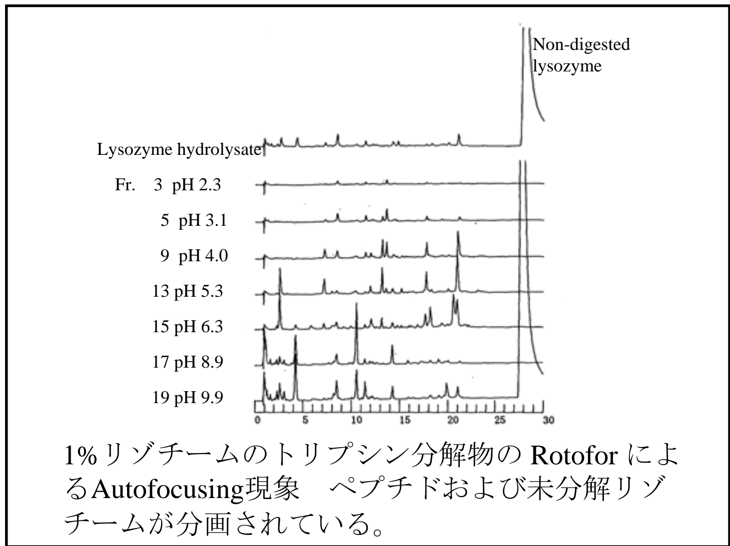
1. 大量分画によるvivoで活性成分の同定

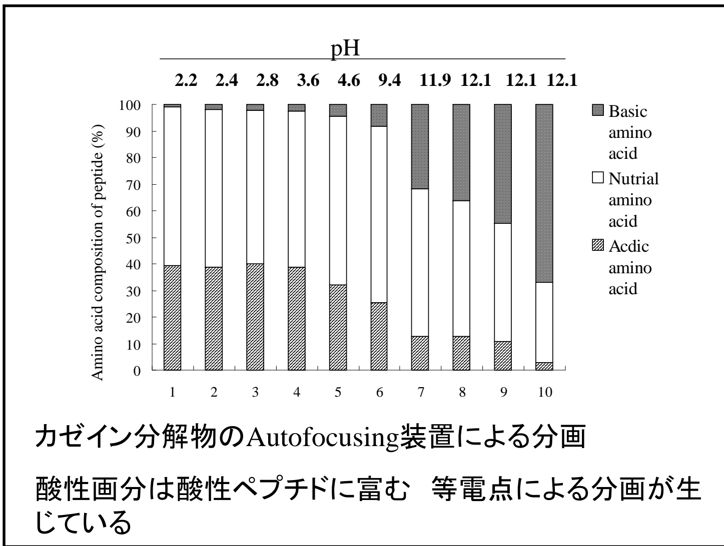
微量のペプチドの分画は可能、しかし、vivoでの評価が可能な量のペプチドを毒性の無い試薬をもちいて、低価格で分画することは困難

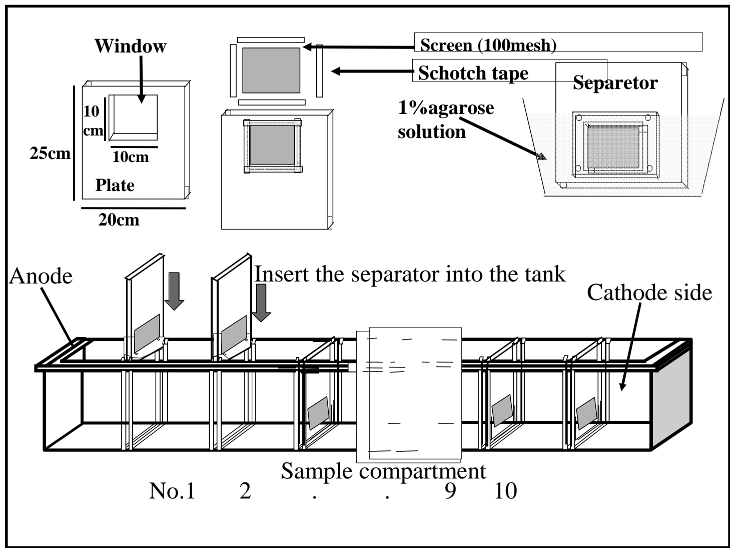
一つの解決として水を溶媒とする大容量Ampholyte-Free 等電点電気泳動法(Autofocusing) を紹介する。

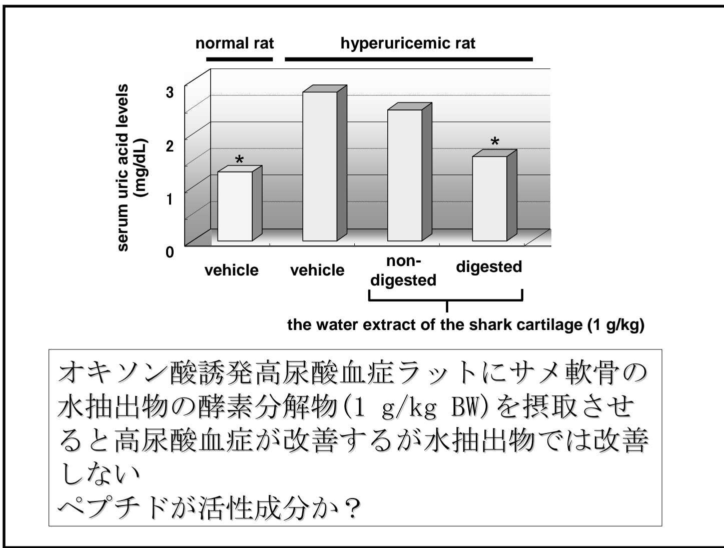
市販調製用等電点電気泳動装置 Rotofor (50 mL sample cell; Bio-Rad)を用いてペプチドのAutofocusing現象を確認




Non-digested
lysozyme
Lysozyme hydrolysate
Fr. 3 pH 2.3
5 pH 3.1
9 pH 4.0
13 pH 5.3
15 pH 6.3
17 pH 8.9
19 pH 9.9
0 5 10 15 20 25 30
1% リゾチームのトリプシン分解物の Rotofor によるAutofocusing現象　ペプチドおよび未分解リゾチームが分画されている。


pH
2.2 2.4 2.8 3.6 4.6 9.4 11.9 12.1 12.1 12.1
Amino acid composition of peptide (%)
0 10 20 30 40 50 60 70 80 90 100
1 2 3 4 5 6 7 8 9 10
Basic amino acid
Nutrial amino acid
Acdic amino acid
カゼイン分解物のAutofocusing装置による分画
酸性画分は酸性ペプチドに富む　等電点による分画が生じている


Window
10 cm
10cm
25cm
Plate
20cm
Screen (100mesh)
Schotch tape
Separetor
1%agarose solution
Anode
Insert the separator into the tank
Cathode side
Sample compartment
No.1 2 . . 9 10


normal rat
hyperuricemic rat
serum uric acid levels (mg/dL)
0 1 2 3
vehicle
vehicle
non-digested
digested
the water extract of the shark cartilage (1 g/kg)
オキソン酸誘発高尿酸血症ラットにサメ軟骨の水抽出物の酵素分解物(1 g/kg BW)を摂取させると高尿酸血症が改善するが水抽出物では改善しない
ペプチドが活性成分か？

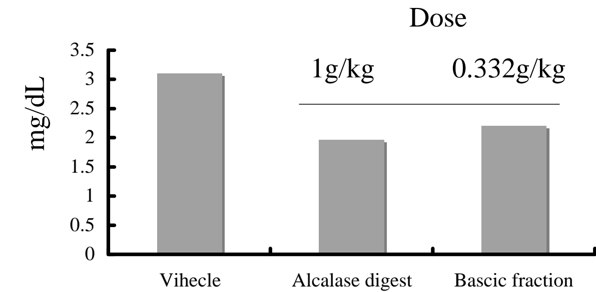
可能性のあるメカニズム

•尿中への尿酸排出促進(ー)

•食事中のプリン体の吸収阻害(ー)

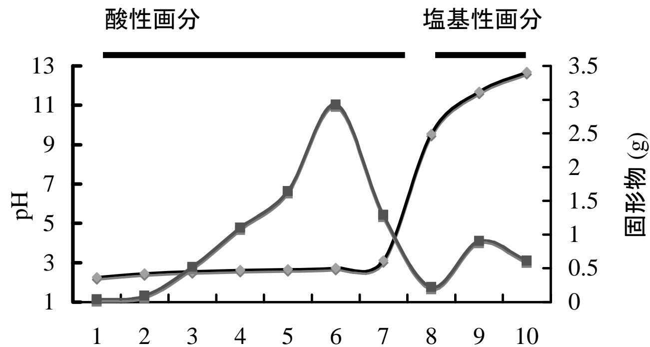
•尿酸合成酵素(キサンチンオキシダーゼ)の阻害 (in vitroではー)

メカニズムが不明　　In vitro
のアッセイができない

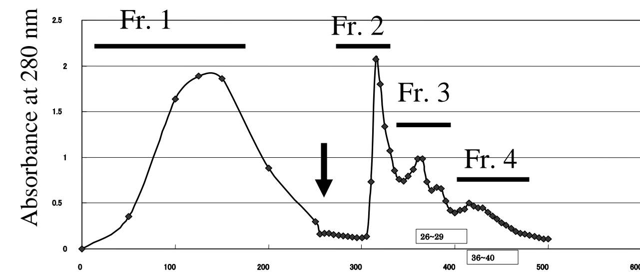


各画分を酵素分解し、動物に投与

塩基性画分は少量で尿酸低下作用を持つ

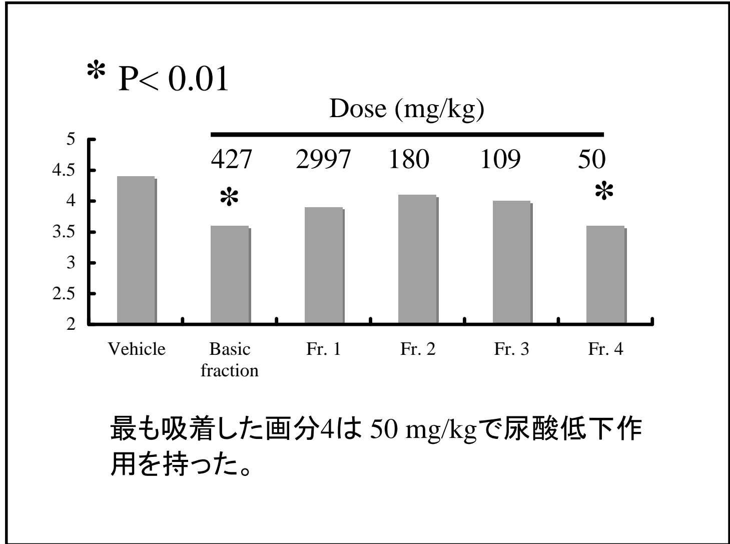


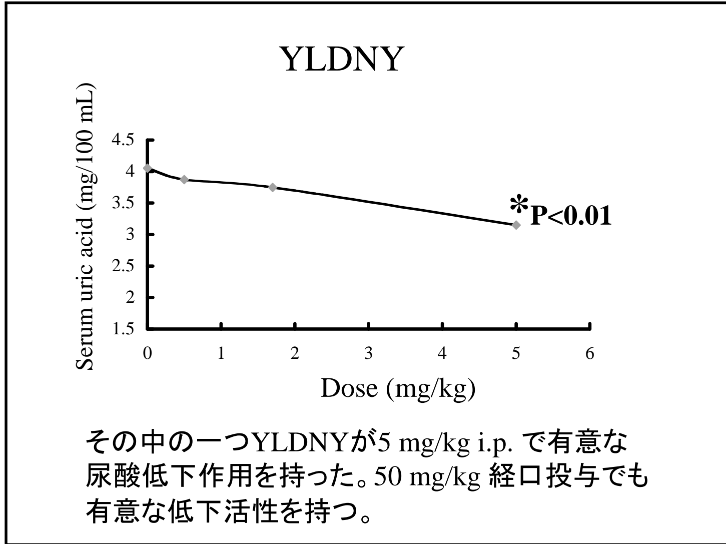
Autofocusing (5 L)によるサメ軟骨の水抽出物の分画

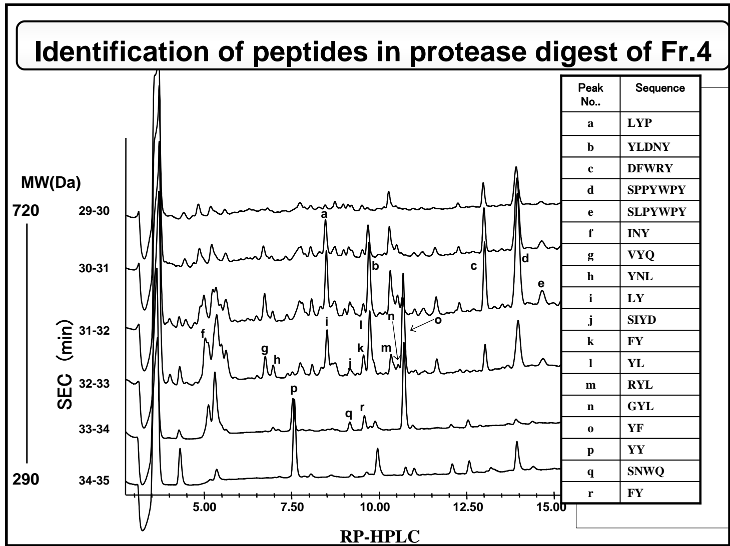


塩基性画分の分取用逆相クロマトグラフィーによる分画

矢印からアセトニトリルの濃度勾配開始







Peak No..	Sequence
a	LYP
b	YLDNY
c	DFWRY
d	SPPYWPY
e	SLPYWPY
f	INY
g	VYQ
h	YNL
i	LY
j	SIYD
k	FY
l	YL
m	RYL
n	GYL
o	YF
p	YY
q	SNWQ
r	FY

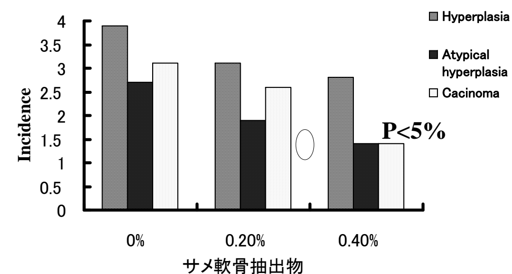
- 従来の in vitroのスクリーニングに基づくアプローチではこのペプチドの同定は不可能
- Vivoでの評価に基づく分画により活性ペプチドの同定とそのメカニズムが解明できた
- 現在、vivoでの評価を基に活性性ペプチドの同定を行っている

鮫軟骨の摂取によるがんの進行抑制

鮫軟骨中に血管新生を抑制し、MMPを抑制する物質が含まれる これは事実。

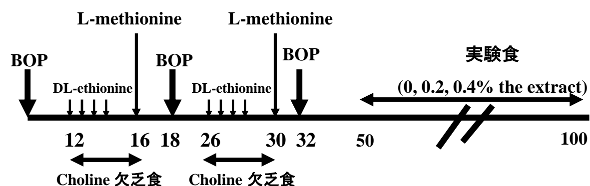
ただし、これは経口摂取での効果とは関係がない。

しかし、一方で鮫軟骨の経口摂取によるがんの進行抑制が期待されている。しかし、アカデミアではほとんど信じられていない。



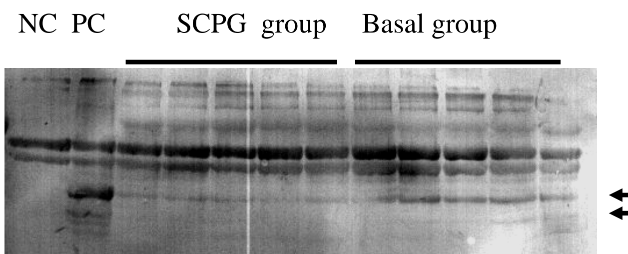
サメ軟骨水抽出物 (SCPG)の経口投与によるがん進行抑制

発がん処理後のがんの進行が、抑制された



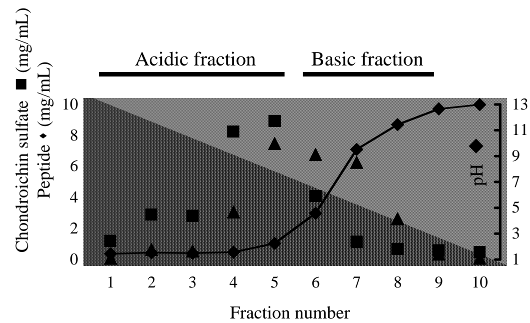
ハムスターを用いた化学誘発膵管がんモデル

BOP; *N*-nitrosobis(2-oxopropyl)amine



担がんハムスターの血清のMMP-9阻害活性

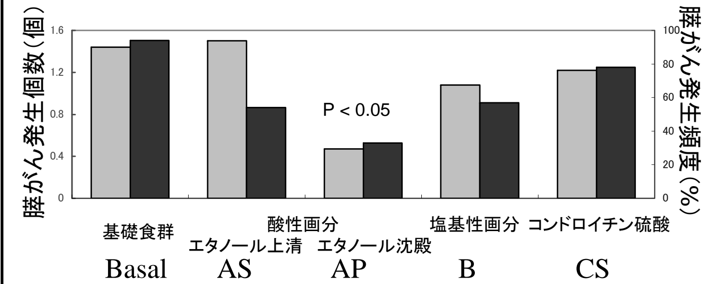
SCPG投与でMMP-9 阻害活性が見かけ上上昇



Autofocusingによる鮫軟骨水抽出物の分画

酸性画分はコンドロイチン硫酸を含む

酸性画分は75%エタノールで沈殿

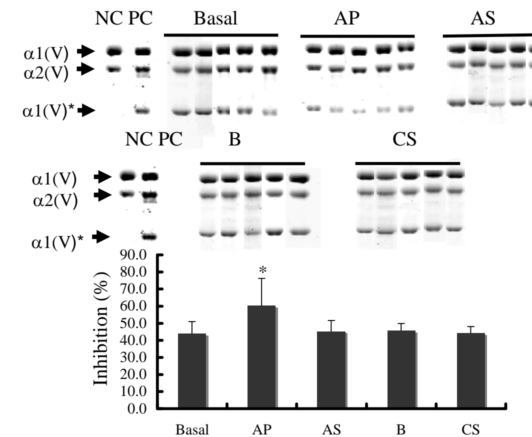


膵がんの進行抑制はAP:プロテオグリカン画分に認められた。

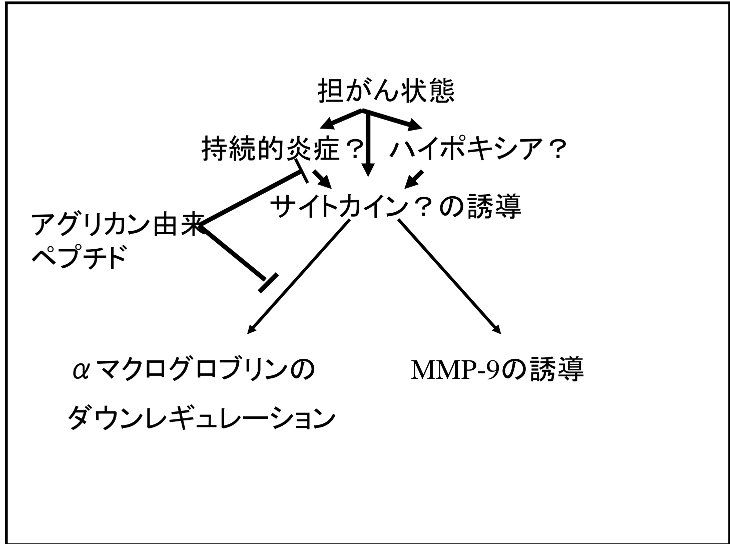
AP: アグリカン様タンパク質を持つプロテオグリカンと少量のコラーゲンペプチド

AS:低分子コンドロイチン硫酸と酸性コラーゲンペプチド

B: 塩基性コラーゲンペプチド



血清中のMMP-9 阻害活性もAPの摂取で増加している。



本プロジェクトは以下のエピソードから始まる

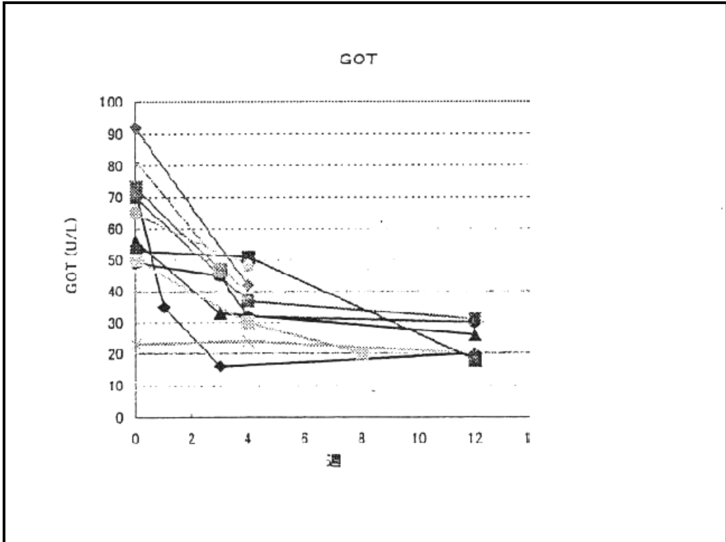
Jpn Pharmacol Ther (薬理と治療) vol. 32 no. 7 2004

Out-hospital Patients with Hyperlipidemia and Hepatitis with Various Backgrounds Improved by Wheat Protein Hydrolysate (Glutamine Peptide) Administration

■

Noboru Horiguchi[1], Hiroshi Horiguchi[1] and Yoshio Suzuki[2]

グルテン酵素分解物中の肝炎抑制ペプチドの同定

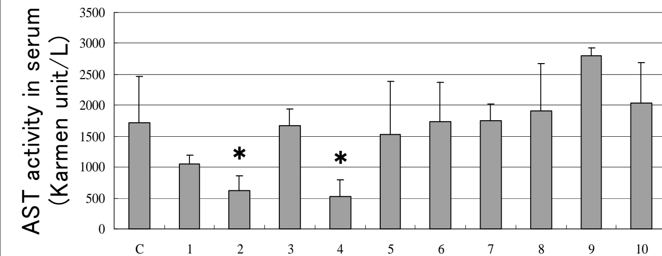


実験

ガラクトサミン誘発肝障害に対する保護効果

WGH中のペプチドを分画し、いずれの画分がガラクトサミン誘発肝障害を抑制するかを検討

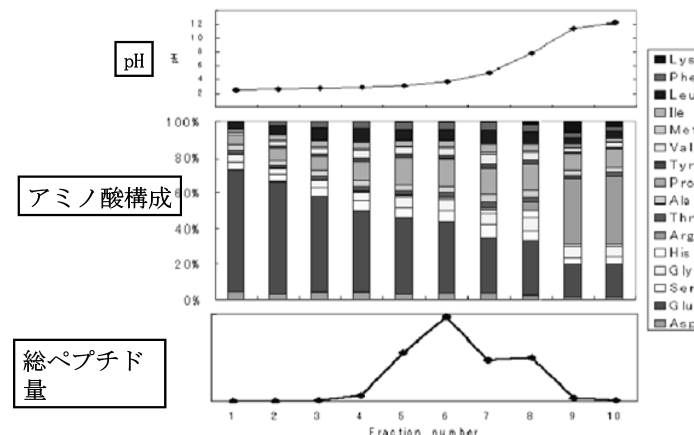
D-ガラクトサミン誘発肝障害モデルラットでのAutofocusing分画物投与による肝障害抑制効果

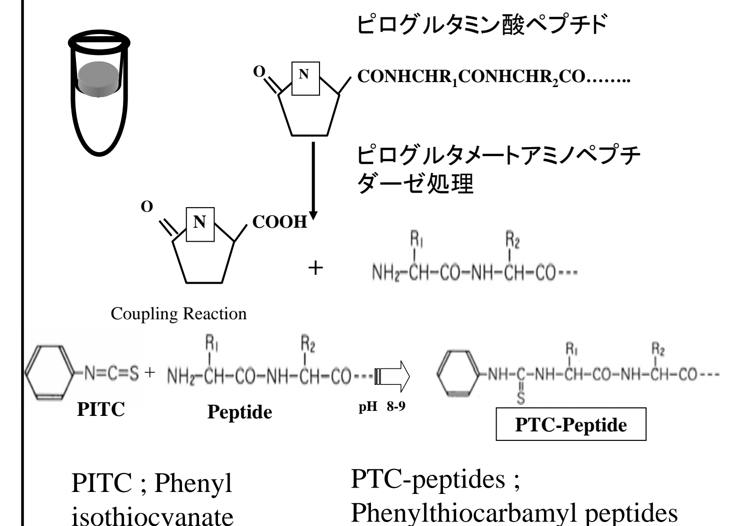


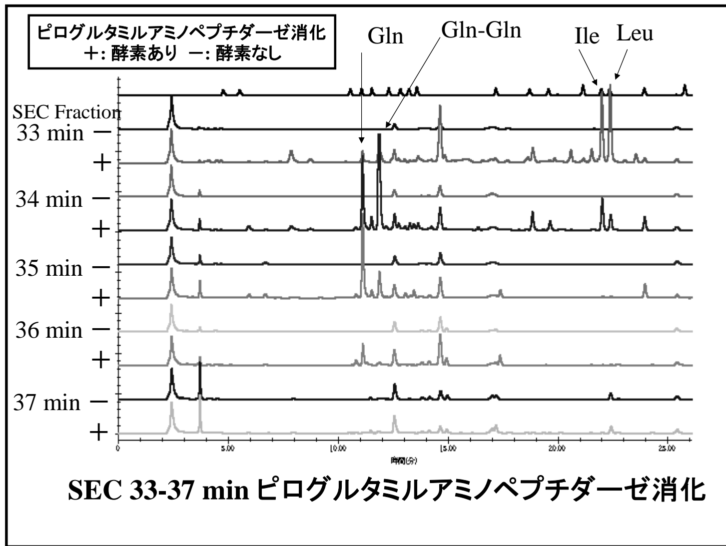
Fraction number	1	2	3	4	5	6	7	8	9	10
投与量(mg/kg体重)	50	8	50	100	100	100	100	100	100	100

（千葉大学園芸学部　真田先生より提供）

各FractionのpH、アミノ酸構成、総ペプチド量

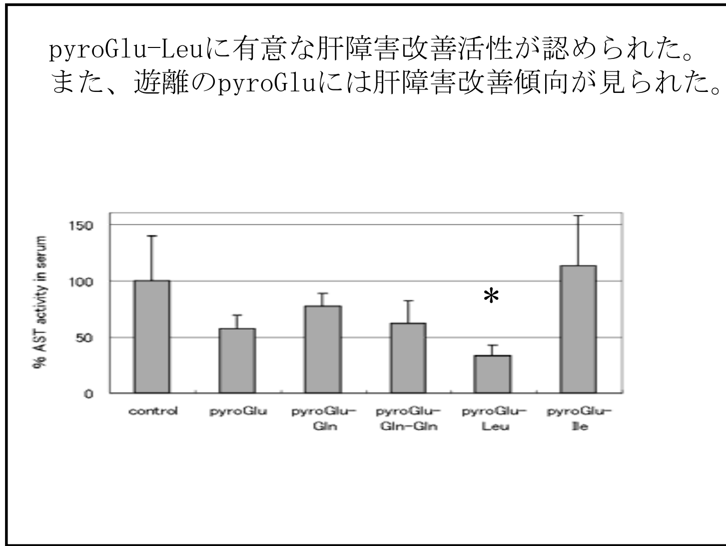


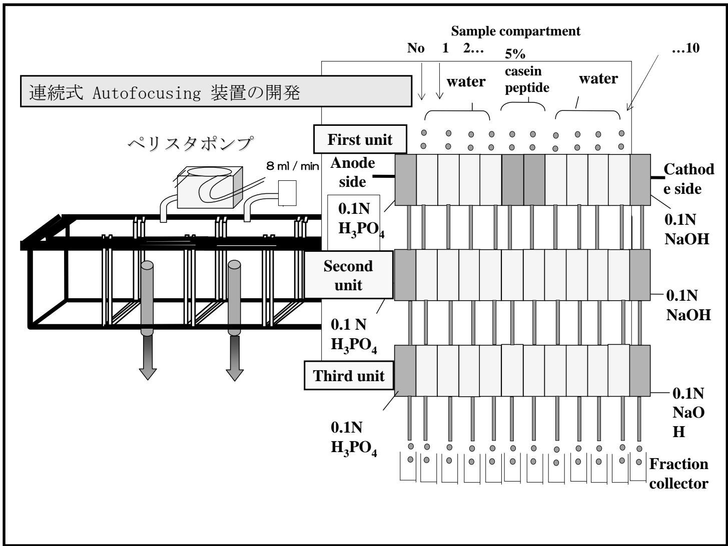


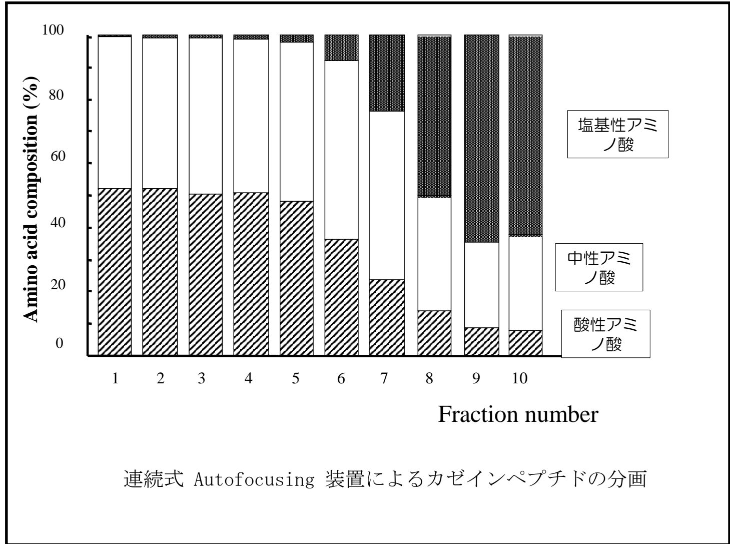


Autofocusing 装置により分画した画分の経口摂取により活性画分を同定してゆき、その中のペプチドを分離同定する。構造情報からペプチドを化学合成し、活性ペプチドを同定する Activity-guided fractionationにより消化・吸収を考えた活性ペプチドの同定が可能である。

またAutofocusing法は機能性成分の濃縮にも利用可能であると考える。







連続式 Autofocusing 装置によるカゼインペプチドの分画

Autofocusingは連続分画も可能であり、食品加工技術としても利用可能であると考えている。

Bioactive peptides—large-scale preparation

Kenji Sato and Kaori Hashimoto

Several scientific studies have revealed that ingestion of some enzymatic hydrolysates of food proteins, namely, mixtures of peptides, produces various beneficial activities beyond basic nutritional values. Moderation of hypertension and hyperlipidemia is considered as one of these beneficial effects.

The emerging market for nutraceuticals and functional foods is stimulating the production of enzymatic hydrolysates of milk, animal, fish, egg, and plant proteins on an industrial scale. Growing worldwide interest in biodiesel and bioethanol production have encouraged consideration of possible added value to be obtained from the by-products arising from their manufacture. The production of bioactive peptide fractions from protein by-products is one attractive approach.

The identification of an active peptide from a mixture of peptides is the initial step required in determining its potential health beneficial properties. In most cases, the active peptides are tentatively identified by high-performance liquid chromatography (HPLC) separation and *in vitro* assays using enzyme and cell culture systems. Unlike many other functional substances, however, peptides are susceptible to degradation during the process of digestion and lose their desired activity. Therefore, the beneficial effects determined *in vitro* cannot be directly linked to those present following digestion. The potential activity of the peptides must be evaluated through feeding experiments.

Although liquid chromatography (LC) is the most powerful tool for isolation of peptides, it is a relatively expensive system for large-scale preparations, especially for the first purification step. In addition, some solvents frequently used in LC, such as methanol, acetonitrile, and trifluoroacetic acid are harmful, but peptide fractions obtained with low selective techniques such as filtration have been used for feeding experiments. Nonetheless, a large-scale, low-cost, and biocompatible procedure for peptide fractionation is needed.

BATCH TYPE AUTOFOCUSING OF BIOACTIVE PEPTIDES

We have demonstrated that peptides can be fractionated on the basis of the amphoteric nature (possessing both acidic and basic properties) of sample peptides without adding chemically synthesized ampholines (having the capacity to act either as an acid or a base) by using a laboratory-scale preparative isoelectric focusing apparatus. This approach has been referred to as autofocusing, and has advantages in cost and biocompatibility over LC. Some

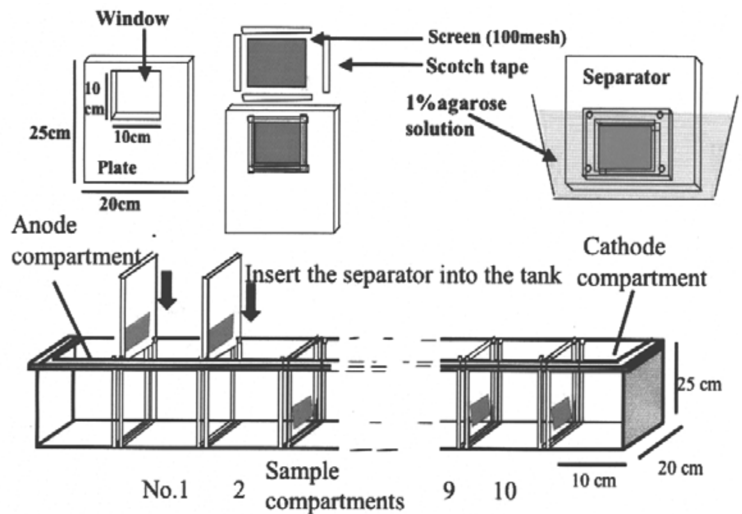


FIG. 1. Schematic drawing of a batch-type autofocusing apparatus. This apparatus can process up to 50 L. Smaller apparatuses with a sample compartment of 5 × 10 × 10 cm or 5 × 7 × 8 cm are also prepared. Total volumes of sample compartments of the three types of apparatus are approximately 50, 5, and 1 L, respectively (Hashimoto et al., *2005).*

preparative matrix-free isoelectric electrophoresis apparatuses have been developed. The main factor that decreases the resolution of matrix-free isoelectric electrophoresis is diffusion of sample by convection current. To minimize the effect of the convection current, a gravity gradient with sucrose and thin-layer focusing cell have been used. These apparatuses can process a sample of less than 1 L in volume. However, further scale-up is considered to be difficult due to the structural nature of the apparatuses. Furthermore, addition of even nontoxic chemicals such as sucrose to form a gravity gradient is not desirable for subsequent feeding experiments. Therefore, we have simply used a thin agarose gel layer (matrix) to avoid diffusion. By using this technique, an autofocusing apparatus which can process up to 5 L has been developed.

Figure 1 shows a schematic drawing of the apparatus. A tank is separated by thin agarose gel layers supported on nylon screens into 12 compartments. The anode compartment is filled with 0.1 N phosphoric acid. The cathode compartment is filled with 0.1 N sodium hydroxide. Other compartments are used as sample compartments and numbered consecutively from the anode side. The cell sample compartments or sample compartments Nos. 5 and 6 are filled with 1–10% peptide solution.

In the latter case, other sample compartments are filled with water. Direct electric current at constant voltage at 300–500 V is

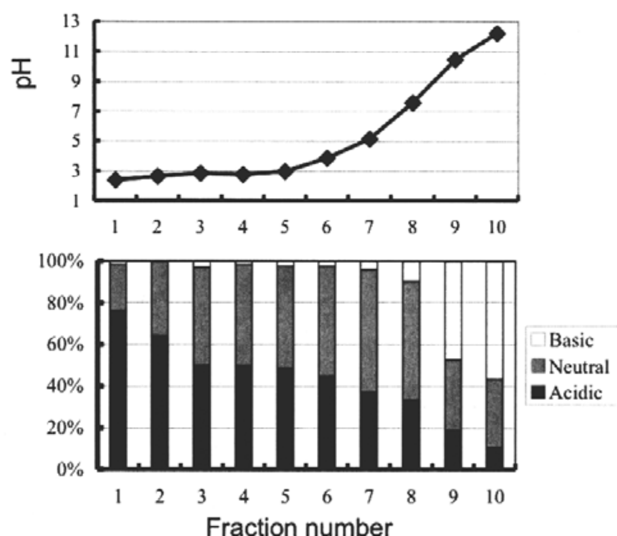


FIG. 2. *Development of pH gradient (upper) and amino acid composition (lower) of each fraction after autofocusing of a commercial enzymatic hydrolysate of wheat gluten by the batch-type apparatus as shown in Figure 1. Amino acid composition is expressed as percentage of acidic, neutral, and basic amino acids.*

applied to the electrodes depending on sample condition. Normally, fractionation is completed after 12–24 h.

Figure 2 shows an example of separation of peptides by the autofocusing apparatus as described in Figure 1. A pH gradient was formed by the autofocusing of an enzymatic hydrolysate of wheat gluten at 500 V for 24 h. Peptides in the acidic and basic fractions are rich in acidic and basic amino acids, respectively, which indicates that peptide separation occurs on the basis of the amphoteric nature of sample peptides.

The pH profile obtained by autofocusing of the same protein hydrolysate may be modified by the concentration of the sample and by the presence of a salt. Fractions with similar pH collected from different batches have essentially the same peptide composition. Thus, autofocusing allows reproducible large-scale separation of peptides. This approach can be considered to be matrix-free electrophoresis. However, separation occurs only in the thin agarose gel layer, which allows separation in a shorter time than conventional large-scale free-zone electrophoresis.

Application of autofocusing at the first purification step has provided a basic peptide fraction, prepared from an enzymatic digest of shark cartilage, that has anti-hyperuricemic activity. With further preparative reversed-phase LC fractionation, we have succeeded in preparing a fraction having significant anti-hyperuricemic activity at 50 mg/kg body weight in an oxonate-induced rat model. (Oxonate inhibits the activity of uricase, the enzyme responsible for the breakdown of uric acid that is produced normally in the body and in excessive amounts in the condition of gout.) Major peptides in the active fraction could be isolated by a series of HPLC and identified without difficulty.

On the basis of the amino acid sequence data, the major peptides in the active fraction were chemically synthesized and used for the feeding experiment. Consequently, a pentapeptide, Tyrosine-Leucine-Aspartic acid-Asparagine-Tyrosine (YLDNY), was identified as the active peptide. The *in vivo* activity-guided large-scale fractionation of peptides by combination of autofocusing and LC followed by identification of the peptides in the active fraction

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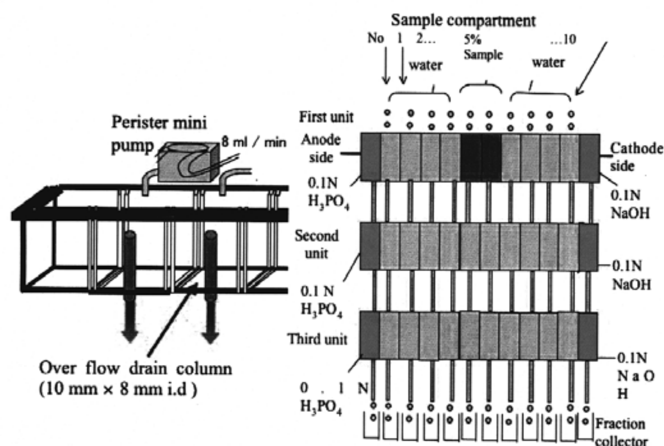

FIG. 3. *Schematic drawing of the continuous type of autofocusing apparatus. In this case, three units of the batch type apparatus with sample compartment (5 × 7 × 8 cm) are used (Hashimoto* et al., *2006).*

would be an effective approach for identification of bioactive peptides following ingestion.

POTENTIAL OF AUTOFOCUSING FOR INDUSTRIAL APPLICATION

The autofocusing system can process a relatively large amount of peptides. The chemicals used, such as sodium hydroxide, phosphoric acid, and agarose, are all food grade. To demonstrate the potential for industrial application, a prototype of a continuous type of autofocusing apparatus has also been developed. As shown in Figure 3, three autofocusing units are connected in tandem. The electrode and sample solutions are continuously delivered to the electrode compartments and sample compartments Nos. 5 and 6, respectively. Water is delivered to other sample compartments. The solution in each compartment in the first unit is continuously delivered to the corresponding compartments of the second and third units. The effluents from the third unit are collected. By using this apparatus, peptides can be continuously separated for 8 hours. This type of continuous apparatus can fractionate larger amounts of peptides using less electric power compared with the batch-type apparatus having the same sample compartment volume. Thus, the continuous-type apparatus is suitable for industrial fractionation of peptides. Crude enzymatic hydrolysates of food proteins frequently show a bitter or odd taste, and most of the constituting peptides are inactive peptides. Separation of the active peptide by autofocusing would improve the taste of peptide-based products by decreasing the amounts of undesired peptides.

CONCLUSION

A mixture of peptides dissolved in water can be fractionated in a preparative isoelectric focusing apparatus on the basis of their amphoteric nature. This approach is referred to as autofocusing and has an advantage in processing cost and biocompatibility over and LC system and can be scaled up. By using thin agarose gel layers to prevent diffusion of the sample, both batch and continuous types of autofocusing apparatus have been developed. The batch type autofocusing apparatus enables *in vivo* activity-guided fractionation for identification of bioactive peptide surviving ingestion. The continuous one would enable preparation of bioactive peptide fractions in concentrated form for use as a functional food ingredient. The present approach would be a breakthroughs not only in basic research but also in industrial processing for development of peptide-based functional foods.

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Isoelectric Focusing

The technique of Isoelectric Focusing is used routinely to separate molecules based on differences in their electric charge, and has found particular application in the separation of proteins and peptides. It is a type of zone electrophoresis, usually performed in a gel such as polyacrylamide, starch, and agarose that takes advantage of the fact that a molecule's electric charge changes with the pH of its environment.

The separation takes place over a medium that has a pH gradient (usually created by aliphatic ampholytes possessing both acidic and basic properties). Passage of an electric current through the medium creates a "positive" anode and a "negative" cathode at each end of the gel and allows negatively charged molecules to migrate through the pH gradient toward the anode and the positively charged particles toward the cathode. As the molecule moves toward the pole opposite of its charge the pH gradient will cause reduction in the degree of charge until a net charge of zero is reached, at which point migration of the molecule will cease.

The particular pH at which a molecule possess a net zero electric charge is its Isoelectric Point, or pI. Many molecules show minimal solubility at their pI. An environment with pH value <pI will result in a molecule carrying a net positive charge; with pH value >pI there will be a net negative charge. Isoelecric focusing can resolve proteins and peptides that differ in pI value by as little as 0.01.

Proteins and peptides owe their ability to carry an electric charge due to varying ionizable groups on the constituent amino acids that reflect their particular composition. Further background information is available online at netlink: http://instruct1.cit.cornell.edu/Courses/biobm330/protlab/IEF.html. ■

information

■ Xiao, Z., T.P. Conrads, D.A. Lucas. G.M. Janini, C.F. Schaefer, K.H. Buetow, H.J. Issaq, and T.D. Veenstra, Direct Ampholyte-free Liquid Phase Isoelectric Peptide Focusing, Application to the Human Serum Proteome, *Electrophoresis 25*:128–133 (2004).

■ Hashimoto, K., K. Sato, Y. Nawa, Y. Nakamura, and K. Ohtsuki, Development of a Large-scale (50 L) Apparatus for Ampholyte-free Isoelectric Focusing (autofocusing) of Peptides in Enzymatic Hydrolysate of Food Proteins, *J. Agric. Food Chem. 53*:3801–3806 (2005).

■ Hashimoto, K., K. Sato, Y. Nakamura, and K. Ohtsuki, Development of Continuous Type Apparatus for Ampholyte-free Isoelectric Focusing (autofocusing) of Peptides in Protein Hydrolysates, *J. Agric. Food Chem. 54*:650–655 (2006).