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Effect of Phytate on the Protein digestibility and Availability *in vitro* of Calcium, Iron and Zinc in Soymilk Treated with Phytase

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ABSTRACT

This study was to examine the effect of phytate on the protein digestibility and calcium, iron and zinc availability in phytase treated soymilks digested with pepsin and pepsin-pancreatin *in vitro*. Also, the binding between phytate and protein in soymilks was investigated by means of SDS-PAGE. The content of phytate in soymilk was reduced by phytase treatment. As the content of phytate decreased, the protein digestibility increased in soymilk treated with the digest enzymes *in vitro*. The reduction of phytate content in soymilk improved the availability of all of calcium, iron and zinc. Although the availability of calcium increased, the amount of change was small. The phytate reduction increased most the availability of iron. A number of bands of high molecular weight protein in soymilk disappeared in SDS-PAGE by lowering the phytate content with phytase treatment on soymilk.

KEY WORDS : soymilk · phytate · phytase · protein digestibility.

Introduction

Soybeans contain phytic acid, an antinutritional factor which is a cause of lowering the protein digestibility and the bioavailability of minerals. When soybeans are processed, phytic acid forms the complexes of phytate-protein, phytate-mineral or phytate-mineral- protein. This leads to a decrease in the availability of protein and minerals. However, the characteristics and mechanisms of interaction between minerals, protein and phytate are not well known and there are a few studies which degree of phytate content can lower the availability of protein and minerals. Several reports using various soybean products did not show consistent results. It was suggested that the interaction between phytate and minerals is more complicated by the processing treatment procedure[1)2)3)].

When cow's milk is replaced with soymilk, the most limited nutrient is calcium. Because soymilk contains less calcium than milk does, high calcium enrichd soymilk is under the development. However, it was reported that calcium increases the precipitation of an insoluble zinc-phytate complex at intestinal pH[1)]. Furthermore, Nahapetian and Young[2)] reported that the addition of calcium to diets high in phytic acid causes a decrease in phytate hydrolysis and reduces zinc absorption in animals. According to Zemel and Shelef[3)], calcium salts and nonfat dry milk depress the

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iron bioavailability in whole wheat bread and have a less effect on the available zinc. Formation of an insoluble calcium-zinc(iron)-phytate complex was suggested as a possible cause.

The many recent studies of mineral bioavailability have been conducted using an *in vitro* method rather than *in vivo* method. The human balance studies are tedious and subjected to large errors[4]. Animal absorption studies are expensive and present a limitation to apply to humans due to the possibility of species differences in mineral metabolism[5]. Human studies using the extrinsic radiomineral tag method have proven to be very successful[6] but the use of human subjects and the administration of radionuclides to humans are not always possible. *In vitro* methods can be rapid, simple and low in cost and offer an appealing alternative to human and animal studies. Most of them attemp to simulate gastrointestinal conditions[7-9].

This study was conducted to evaluate the protein digestibility and calcium, iron, and zinc availability in *in vitro* digests of soymilk containing various phytate contents due to the results of phytase treatment. An additional objective was to study the binding pattern of a phytate-protein complex by gel electrophoresis.

Materials and Methods

1. Materials and sample preparation

Soybeans (*Glycine max, var.* Jangyup) were purchased from Korean Rural Development Administraion. Phytase(P-1259) was obtained from Sigma Chemical Co. (USA). A procedure by Lee[10]was used for soymilk preparation. The soymilk was adjusted to pH 5.15 with HCl solution. And 0.03g, 0.1g or 0.2g phytase was added to each 50 mL soymilk. After incubation in a shaking waterbath at 55℃ for 5 hr, to stop phytase activity HCl was added to soymilk until its pH became below 2.

The soymilks added 0g, 0.03g, 0.1g and 0.2g phytase were coded as SSM(Standard soymilk), 0.03SM, 0.1SM and 0.2SM respectively.

2. Phytate determination

Phytate was determined using the method described by Latta and Eskin[11]. Absorbance at 500nm using spectrophotometer(Hitachi U-1100, Japan) was measured.

3. Simulated digestion and protein digestibility of soymilk

The method of Miller, *et al*[8]. with some modification was used for digestion of samples to determine the minerals availability *in vitro* in soymilk treated with phytase. 20mL of pH 2 HCl solution and 55mg pepsin(Sigma Chemical Co. p-7000) were added to 5mL phytase treated soymilks. The samples were incubated in a 37℃ shaking waterbath for 2 hr. The digestion was then either stopped at this point and the samples centrifuged as described below or the samples were raised to pH 7.0 ± 0.1 with sodium bicarbonate, taking precautions to minimize CO_2 loss. Each sample was mixed with 2.5 mL of a suspension containing 0.5 % pancreatin (Sigma Chemical Co. P-1750) and 5 % bile (Sigma Chemical Co. B-8631) and incubated in a 37℃ shaking water bath for 2 hr. Immediately following digestion, samples were centrifuged at 14,000g for 40 min at 4℃.

For protein digestibility determination, *in vitro* method of Knuckles, *et al*[12]. was used. Dialysates after various time periods up to 24 hr for digest enzymes treatment were analyzed for protein concentrations by the method of Lowry, *et al*[13]. using bovine serum albumin as reference. For total protein analysis, the AOAC microKjeldahl procedure[14] and a conversion factor of 6.25 were used.

4. Total and soluble calcium, iron and zinc determination

The sample was ashed overnight at 550℃ in a muffle furnace. After cooling, the ash was dissolved in concentrated nitric acid and heated at 100℃. Then the ash was dissoved in concentrated HCl, diluted to an appropriate volume and analyzed for calcium, iron and zinc using atomic absorption spectrophotometer (GBS904, USA). After digestion, the resulting supernatants were filtered through Whatman #1 paper and analyzed for soluble calcium, iron and zinc using atomic absorption spectrophotometer.

5. Ionizable calcium

To the filtrates, 4 M KCl was added to adjust the background ionic strength and ionic calcium was measured using a calcium ion-selective electrode(Phoenix Electrode Co. CAL 1502, USA).

6. Electrophoresis

For electrophoretic pattern of protein in soymilk treated with phytase SDS-PAGE (sodium dodesyl sulfate-polyacrylamide gel electrophoresis) was performed according to the method of Laemmli[15]. Run was performed at 200 V. Gels were stained in a staining solution containing 0.1% Coomassie blue G-250, 45% methanol and 45% acetic acid and destained in a destaining solution of 10% methanol and 10% acetic acid.

7. Statistical analysis

Each determination was replicated at minimum of eight times. All data were analyzed by analysis of variance and Duncan's Multiple Range test to separate significant means.

Results and discussion

1. Changes in phytate content

The phytate content in the standard soymilk was 0.496%. The effects of phytase concentration and treatment duration on phytate concentration in soymilk are shown in Fig. 1. It indicates that the phytate reduced slowly after 5hr of phytase treatment. Therefore, the soymilks treated phytase for 5hr for determination of protein digestibility and minerals availability were used as the samples. The remained phytate content of 0.03SM (0.03 g phytase treatment in 50 mL standard soymilk) after 5hr incubation was 67.71% of total phytate in the standard soymilk, that of 0.1SM(0.1g phytase treatment) was 50.21% and

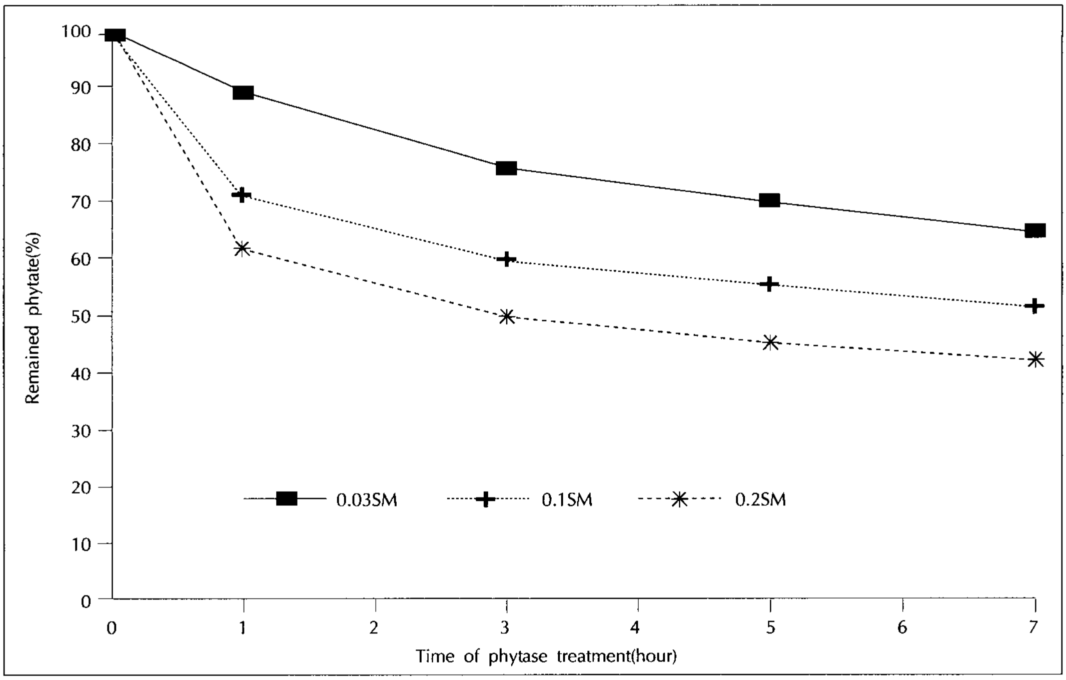

Fig. 1. Effect of concentration and duration of phytase treatment on phytate in soy milk.
0.03SM : 0.03g phytase treated soymilk
0.1SM : 0.1g phytase treated soymilk
0.2SM : 0.2g phytase treated soymilk

that of 0.2SM (0.2g phytase treatment) was 41.46 %.

2. Protein digestibility

Protein digestibilities by pepsin over 24 hr period are given Fig. 2. Low-phytate soymilk protein was more digestible than high-phytate one over 24 hr.

As shown in Fig. 3, the protein digestibilities by pepsin-pancreatin were generally similar to those obtained with pepsin. However, the difference in digestibility among samples by pepsin-pancreatin was smaller than that by pepsin digestion. This is thought to be due to the difference of the phytate-protein interactions at different pH. The phytate-protein interactions are ionic at low pH and mediated by cations through the formation of phytate-cation- protein complexes at high pH[16]. It is suggested that the different protein complex forms at pH 2 and pH 7 may lead to the differnt digestibility.

3. Changes in calcium, iron and zinc contents

In this study, the total calcium, iron and zinc levels in 1mL of soymilk were 271.7㎍, 16.9㎍ and 17.3㎍, respectively. The soluble and ionic calcium levels in digests are shown in Table 1. The soluble calcium levels in pepsin-pancreatic digests were about half of those in peptic digests. The most likely explanation for these reductions is the formation of insoluble calcium complexes(amino acid/polypeptide-calcium-zinc-phytates, calcium-zinc-phytates) at a neutral pH. This concept is further supported by the study of the mineral solubility depending on pH by Kantha, *et al*[17]. And this is partly due to the formation of insoluble calcium and zinc hydroxide during neutralization.

In peptic digests, approximately 2/3 of the soluble calcium was in the ionic fraction. There was no significant amount of ionic calcium in pepsin-pancreatic digests. The binding to amino acid and small peptide

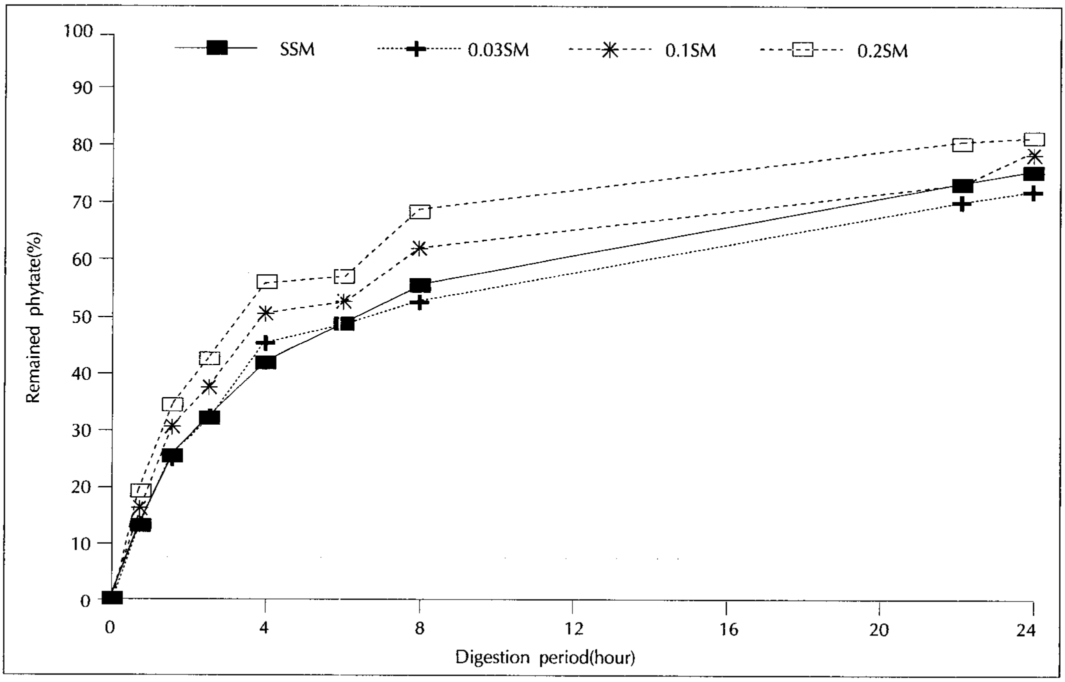

Fig. 2. Protein digestion profile upon pepsin digestion of soymilks treated with phytase.
SSM : standard soymilk
0.03SM : 0.03g phytase treated soymilk
0.1SM : 0.1g phytase treated soymilk
0.2SM : 0.2g phytase treated soymilk

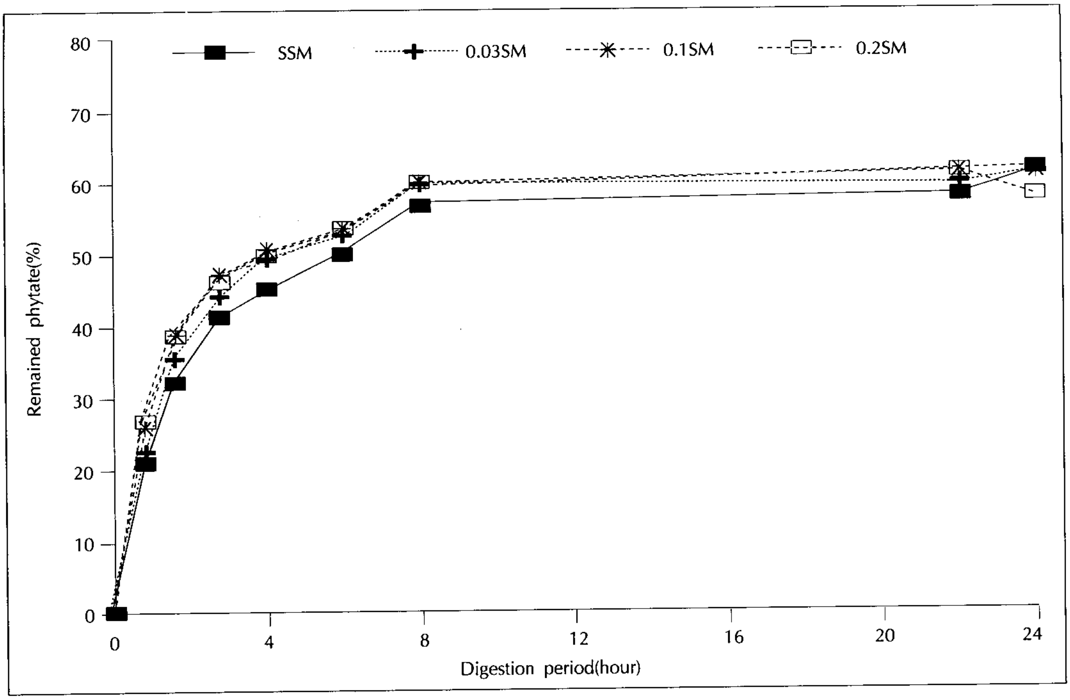

Fig. 3. Protein digestion profile upon pepsin-pancreatin digestion of soymilks treated with phytase.
SSM : standard soymilk
0.03SM : 0.03g phytase treated soymilk
0.1SM : 0.1g phytase treated soymilk
0.2SM : 0.2g phytase treated soymilk

Table 1. Contents of soluble and ionic calcium in digests of soymilks (μg Ca/ml soymilk)

Treatment	Soluble Ca		Ionic Ca	
	pepsin	pepsin-pancreatin	pepsin	pepsin-pancreatin
SSM	206.1^{a}	96.6^{a}	147.5^{a}	n.d
0.03SM	234.3^{a}	97.7^{a}	152.2^{b}	n.d
0.1SM	236.6^{a}	107.0^{a}	158.4^{bc}	n.d
0.2SM	237.7^{a}	118.3^{b}	164.4^{c}	n.d

n.d. : non detectable
Means with the same letter on the row are not significantly different, $p<0.05$.
SSM : standard soymilk
0.03SM : 0.03g phytase treated soymilk
0.1SM : 0.1g phytase treated soymilk
0.2SM : 0.2g phytase treated soymilk

Table 2. Contents of soluble iron and zinc in digests of soymilks (ppm/ml soymilk)

Treatment	Fe		Zn	
	pepsin	pepsin-pancreatin	pepsin	pepsin-pancreatin
SSM	1.52^{a}	2.09^{a}	5.91^{a}	3.41^{a}
0.03SM	1.73^{a}	5.46^{b}	6.01^{a}	4.36^{b}
0.1SM	3.32^{b}	7.03^{c}	6.38^{a}	4.87^{bc}
0.2SM	4.82^{c}	7.11^{c}	6.54^{a}	5.13^{c}

Means with the same letter on the row are not significantly different, $p < 0.05$.
SSM : standard soymilk
0.03SM : 0.03g phytase treated soymilk
0.1SM : 0.1g phytase treated soymilk
0.2SM : 0.2g phytase treated soymilk

and the reduction of solubility of ionic calcium at a neutral pH might lead to that result. There was more ionic calcium in peptic digest of low phytate soymilk. Although the measurement of ionic calcium in pepsin-pancreatic digests did not provide an indication of the relative predictive value of calcium bioavailability, phytate seemed to have an adverse effect on the availability of calcium, when the calcium is absorbed in the upper part of intestine at a acidic pH. Since the increasing rate of soluble calcium was much smaller than the reduction rate of phytate, other factors may have more effect on the availability of calcium than phytate does.

The effects of digestion on iron solubility are shown in Table 2. As the content of phytate reduced, the soluble iron increased greatly. Contrary to calcium and zinc, there was the more amount of soluble iron in pepsin-pancreatic digests than in peptic ones. Kantha *et al*[17]. reported that iron showed the maximum solubility between pH 6 and pH 7. And in this investigation, this effect was consistently found. When soluble iron in foods subjected to *in vitro* gastric or gastrointestinal digestion is used as the index of potential iron bioavailability, the availability of iron is reduced greatly by phytate.

Soluble zinc in pepsin-pancreatic digests shown in Table 2 was less than in peptic digests and this result consists with the study by Kantha *et al*[17]. Also, the reduction in zinc solubilities resulting from pancreatic digestion of soymilks initially treated with pepsin at pH 7.0 is a reflection of the formation of pepsin-zinc-phytate and insoluble complexes with bile salts[18][19]. Soluble zinc was not significantly different among pep-

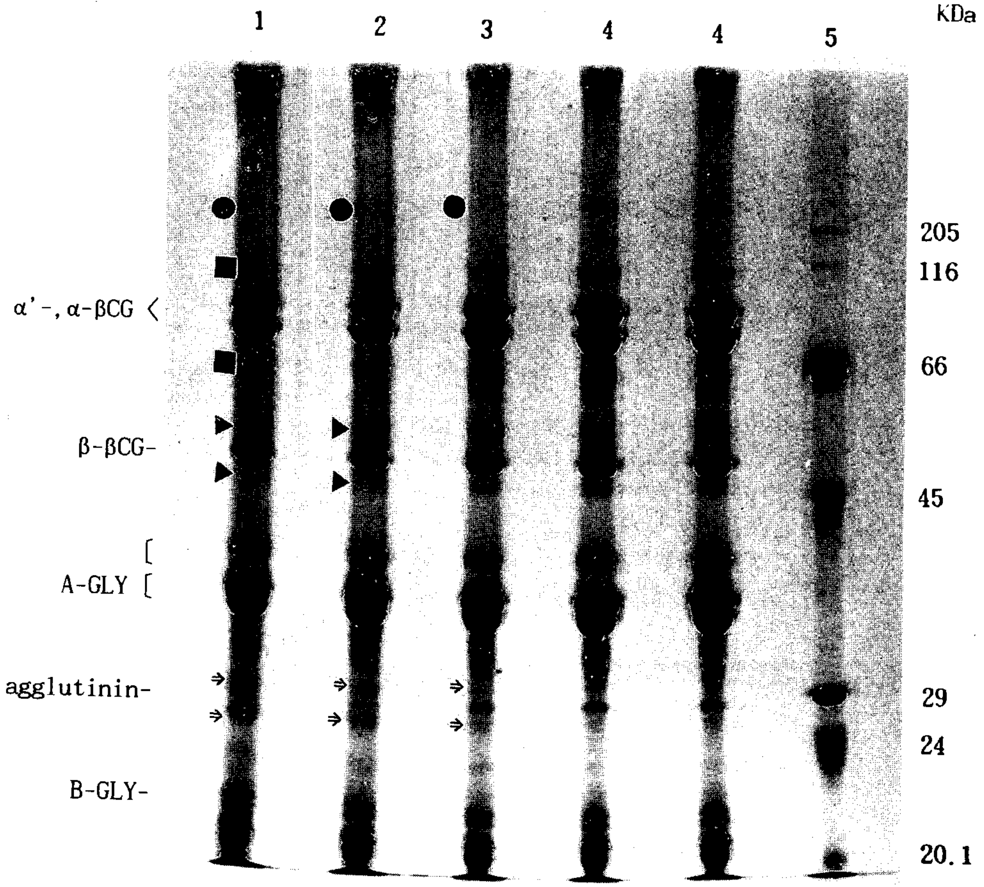

Fig. 4 SDS-PAGE pattern of the protein of phytase treated soymilks

1, 0.2SM ; 0.2g phytase treated soymilk
2, 0.1SM ; 0.1g phytase treated soymilk
3, 0.03SM ; 0.03g phytase treated soymilk
4, SSM ; standard soymilk 5, standard proteins
●, bands disappeared in all phytase treated soymilks
▶, bands disappeared in 0.1SM and 0.2SM
■, bands disappeared in only 0.2SM
⇒, new bands appeared in all phytase treated soymilks
A-GLY : acidic glycinin polypeptides
B-GLY : basic glycinin polypeptides
β-βCG : β subunit of β-conglycinin
α'- and α-βCG : α' and α subunits of β-conglycinin

tic digests. However, in pepsin-pancreatic digests, there was a considerable difference between standard soymilk and phytase treated soymilks. Considering zinc is absorbed mainly in the lower part of intestine, it is suggested that phytate reduces greatly the availability of zinc.

Electrophoresis

The effect of phytate on the electrophoretic pattern of soymilk protein is shown Fig. 4. Fraction components were identified by comparison to standard protein and identification in the literature$^{20)21)22)}$. All samples show several bands corresponding to the two major soybean proteins, glycinin and β-conglycinin. And they also show unidentified bands which may be modified protein resulting from the heat treatment. In the phytase treated soymilks, the band of some high molecular weight proteins become unclear and the band of low molecular weight proteins are appeared. The bands corresponding to β-conglycinin become dimmer, while the bands corresponding to glycinin are little changed. This is consistent with the observation by Brooks and Morr$^{23)}$ that 7S soy protein(β-conglycinin) may be associated with phytate. The bands corresponding to agglutinin become dimmer in the phytase treated soymilks in agreement with Honig and wolf$^{24)}$. Many unidentified bands also changed and disappeared in the phytase treated soymilks. This study indicates associations of soymilk proteins with phytic acid and minerals or other soybean components and formation of high molecular weight complexes.

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=국 문 초 록=

피티아제를 처리한 두유의 단백질 소화율과 칼슘, 철, 아연의 유동도에 대한 피트산의 효과

황 인 경 · 이 묘 용

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본 연구는 phytase를 처리하여 phytate 함량을 낮춘 두유에 펩신과 펩신-판크레아틴 효소를 실험관 방법으로 작용시킨 후 두유 단백질의 소화율과 Ca, Fe, Zn의 유용성을 측정하였으며 두유 단백질과 phytate의 결합양상을 살펴보았다. 두유의 phytate 함량은 phytase 처리에 의해 낮아졌다. 소화효소 처리시 phytate 함량이 낮은 두유의 단백질 소화율은 높아졌으며 Ca, Fe, Zn의 유용성은 증진되었고 특히 Fe의 유용성이 가장 크게 증가되었다. 두유의 phytase 처리에 의해 고분자 단백질 밴드의 소멸이 SDS-PAGE에 의해 관찰되었다.