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ISOLATION AND IDENTIFICATION OF PEPTIDES FROM SOY 11S-GLOBULIN WITH HYPOCHOLESTEROLEMIC ACTIVITY

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A peptide with hypocholesterolemic activity was isolated by HPLC from the pepsin hydrolysate of 11S-globulin. Its molecular weight (755.2 Da) and amino-acid sequence (Ile-Ala-Val-Pro-Gly-Glu-Val-Ala) were established. The hypocholesterolemic effect was determined by analysis of bile-acid binding and the percent inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase in vitro. The lowering of the cholesterol content is explained by bile acids bound to hydrolysate peptides shielding them from reabsorption and stimulating the transformation of cholesterol in blood plasma.

Key words: soy 11S- and 7S-globulins, pepsin hydrolysate, hypocholesterolemic effect.

Numerous investigations [1] have shown that the relatively low risk of coronary heart disease in Asian countries is related to the use of soy protein as a food source. The use of products with soy protein at least four times per week decreases the risk of heart disease by 22% [2]. The cholesterol content is reduced more and the binding of bile acids is more effective by hydrophobic fractions resulting from hydrolysis of 11S- and 7S-globulins than by the action of natural soy protein [3].

We investigated the hypocholesterolemic properties of globulins from soy beans and the isolated peptides that are responsible for this action. Reserve proteins are globulins and make up about 50-90% of the total protein content of soy beans [4]. Globulins in soy beans are grouped into two types, 7S- and 11S-globulins, according to their sedimentation coefficients. Soy 7S-globulin consists of three subunits with different physicochemical properties. Soy 11S-globulin (glycinin hexamer) of molecular weight (MW) 300-380 kDa is a spherical globule. Each subunit consists of an acidic polypeptide A of MW 35 kDa and a basic polypeptide B of MW ~20 kDa. The quaternary structure of glycinin is stabilized by hydrophobic and electrostatic interactions and disulfide bonds between the acidic and basic polypeptides [5].

The peptide Leu-Pro-Tyr-Pro-Arg (LPYPR) was previously isolated from soy-protein glycinin and determined to be a hypocholesterolemic agent [6]. It has been demonstrated that soy 11S-globulin has a higher hypocholesterolemic effect than native soy protein, 7S-globulin, and casein. Other researchers [7] also observed in the trypsin hydrolysate of glycinin a peptide with a hypocholesterolemic effect, isolated it, and determined the amino-acid sequence as Leu-Pro-Tyr-Pro (LPYP).

The goal of our research was to study the interaction of peptides from the pepsin hydrolysate of 11S-globulin with bile acids, to explain the reduced cholesterol content in blood plasma, and to isolate a peptide with a high hypocholesterolemic effect.

7S- and 11S-globulins were isolated as before [8]. The content and homogeneity of the 7S- and 11S-proteins were determined by electrophoresis in polyacrylamide gel (10-15%) and estimated as 90 and 93%, respectively, by densitometric gel analysis.

11S-Globulin was hydrolyzed by five proteases: trypsin and pepsin and the proteases from *Aspergillus saitoi*, *A. oryzae*, and a *Rhizopus sp.*. Table 1 shows that the protein-nitrogen content and degree of hydrolysis were greatest if pepsin was used. It should be considered that pepsin is less specific than trypsin and may give peptides consisting of several amino acids. Pepsin was chosen for the hydrolysis of glycinin for this reason. The resulting peptide fractions were investigated for their hypocholesterolemic effect.

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TABLE 1. Protein-Nitrogen Content in Protein Hydrolysate and Degree of Hydrolysis of Protein by Various Proteases after 24 h Determined by Biuret

Enzyme	mg protein N/mg whole protein	Degree of hydrolysis, %
Trypsin	0.0006	9.4
Pepsin	0.0035	52.1
Protease from *Aspergillus saitoi*	0.0017	24.7
Protease from *Aspergillus oryzae*	0.0024	34.7
Protease from *Rhizopus sp.*	0.0025	37.5

Note. All proteases were obtained from Sigma.

TABLE 2. Inhibition of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (HMGCoAR) Activity by Fractions A, B, C, and D

Fraction	HMG CoA reductase activity, pmol/min/mg protein	Inhibition rate, %
Control	4.55	
A	3.15	31
B	2.49	45
C	2.55	43
D	2.30	50

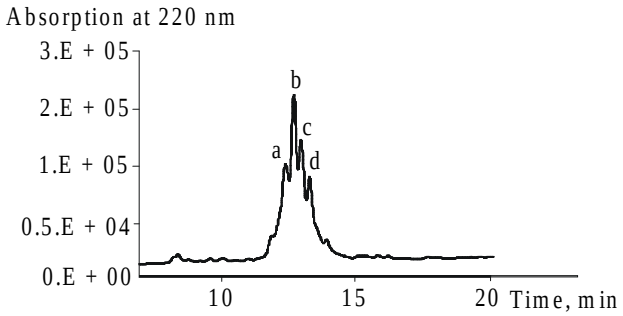

Fig. 1

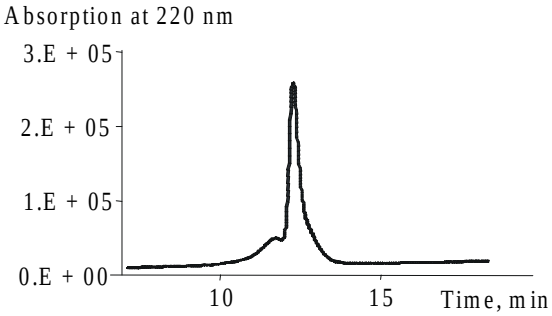

Fig.2

Fig. 1. Rechromatography of hypocholesterolemic peptide fraction D.
Fig. 2. Rechromatography of peptide fraction b.

The pepsin hydrolysate of glycinin was purified and separated by chromatography (RP-HPLC, Jasco, Tokyo, Japan) using a C18 column.

The pepsin hydrolysate of glycinin gave several fragments that were separated by reversed-phase HPLC. The elution profile consisted of a large number of peaks separated into four fractions (A, B, C, D). The corresponding fractions were collected and evaluated for their hypocholesterolemic activity. The hypocholesterolemic effect of the pepsin-hydrolysate fractions was tested in in vitro investigations to determine the percent inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), which is known to be a key enzyme in cholesterol biosynthesis [9]. The action of HMG-CoA reductase on fresh microsomes from rat liver was determined by the literature method [10]. Fraction D had the highest hypocholesterolemic effect (Table 2).

Fraction D was rechromatographed over a C18 column using various gradient elutions. The elution profile of fraction D consisted of four peaks (a, b, c, d) (Fig. 1). Each peak, corresponding to the four peaks, was collected and tested for hypocholesterolemic activity. The rates of inhibition (HMG-CoAR) of peaks a, b, c, and d were 37, 75, 46, and 28%, respectively. According to the results, peak b was the strongest inhibitor of HMGR. It was rechromatographed using several columns with variable gradient conditions (Fig. 2) in order to isolate a peptide with hypocholesterolemic activity.

TABLE 3. Binding in vitro of Bile Acids by Pepsin Hydrolysate of 11S-Glycinin

Bile acid	Binding of bile acids, mmol/100 mg	Percent binding of bile acids
Cholic	89±0.6	72±0.5
Deoxycholic	81±0.3	65±0.2
Glycocholic	8±0.6	6±0.4
Taurocholic	9±1.6	7±1.3

The amino-acid sequence of the isolated peak was determined by Edman degradation [11]. According to the results, peak b contained a peptide with the amino-acid sequence Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA). The theoretical MW of this sequence is 754.9 Da. This agreed well with results from mass spectrometry, which indicated a MW of 755.2 Da. These results confirmed that the isolated homogeneous peptide was pure.

It is known that hypocholesterolemia can accelerate the development of coronary artery disease and cause atherosclerotic damage [11-12]. Decreasing the level of circulating cholesterol decreases the risk of these diseases. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) is an enzyme that controls the rate of cholesterol biosynthesis. According to the results of in vitro studies, the inhibition of HMGR by peak d reduced the cholesterol content.

It is currently hypothesized that bile acids may bind with dietary fiber to reduce the cholesterol content [13]. An investigation of the binding capability of cholic, deoxycholic, glycocholic, and taurocholic acids might give data for the comparative interaction of the pepsin hydrolysate of glycinin in experiments imitating the conditions in the liver and small intestine. A published in vitro bile-binding procedure was used [15]. Bound and free bile acids were separated by centrifugation.

Table 3 lists the data for in vitro binding of conjugated bile acids with glycinin hydrolysate.

According to the results, pepsin hydrolysate binds significantly cholic and deoxycholic acids and less efficiently glycocholic and taurocholic acids. Previous investigations [16] showed that hydrophobic interactions affect the binding of bile acids. The interaction of deoxycholic acid may be stronger than that of cholic acid. A comparison of the interaction of cholic and deoxycholic acids indicates that cholic acid is more capable of binding than deoxycholic acid. This is a result of Phe, Tyr, and Trp units at the peptide terminus in the peptides of the pepsin hydrolysate of 11S-globulin. For cholic acid, the equatorial 3-hydroxyl is more available than the 7- and 12-axial hydroxyls. This may play a role in determining the orientation of the hydrophobic part of the peptide for the best binding.

For deoxycholic acid, which is a dihydroxy bile acid, the binding is due to analogous interactions. Judging from studies of bile-acid salts, salts of glycocholic and taurocholic acids are more strongly involved in ion—ion interactions with peptide fragments. As a result, this changes the orientation of the hydrophobic part of the fragments and reduces the binding.

Thus, digestion of soy 11S-globulin by pepsin produced the hypocholesterolemic peptide IAVPGEVA, which differs from the previously isolated peptide LPYP from the trypsin hydrolysate [7]. The difference in the amino-acid sequence may be explained by the specific activities of trypsin and pepsin. The trypsin hydrolysate typically contains peptides with Lys and Arg units at the N-terminus whereas pepsin cleaves glycinin to form peptides with Phe, Tyr, and Trp at the N-terminus. Both isolated peptides have hypocholesterolemic activity in proportion primarily to the hydrophobic amino acids.

An in vitro analysis of the binding of bile acids is consistent with the hypothesized mechanism according to which dietary fiber lowers the cholesterol content because the binding of bile acids to digested fragments prevents them from being reabsorbed and stimulates cholesterol transformation into bile acids in plasma and the liver [17]. The results suggest that the degree of binding of bile acids and their salts may depend on their orientation for binding the hydrophobic part of cholic and deoxycholic acids.

Our results indicate a positive correlation between the binding of cholic and deoxycholic acids and the hypocholesterolemic effect of the pepsin hydrolysate. Such a correlation is not observed for glycocholic and taurocholic acids. Prior analyses [8] showed that the peptides LPYP, LPYPR, and SPYPR had hypocholesterolemic effects and gave a negative correlation for binding of bile acids. Therefore, the degree of interaction of bile acids is not essential for identifying hypocholesterolemic activity.

Results from in vitro inhibition of HMGR suggest another mechanism for the lowered cholesterol level, namely inhibited HMGR activity is an indicator of a limited rate of cholesterol biosynthesis. This effect in fractions with hypocholesterolemic activity depends on both the peptide content and the difference in the amino-acid sequences of the resulting peptides.

EXPERIMENTAL

Materials. Reagents and solvents were obtained commercially (Sigma Chemical Co., St. Louis, MO, USA).

Isolation of 7S- and 11S-Globulins. The 7S- and 11S-globulins were isolated from defatted soy beans by extraction with water (15-fold excess) adjusted to pH 7.5 with NaOH solution (2 N). The extract was centrifuged at 2000×g for 30 min. The supernatant was treated with anhydrous sodium bisulfite (0.98 g/L). The pH was adjusted to 6.4 with HCl solution (2 N). The solution was stirred, stored on an ice bath overnight, and centrifuged (6500×g, 20 min, 4°C). The insoluble fraction, which contained the 11S-globulin, was washed, centrifuged again under the same conditions, suspended in water, dialyzed overnight, and lyophilized. The soluble fraction was treated with NaCl until the concentration was 0.25 M. The pH of the supernatant was 5.0. After 1 h the insoluble fraction was separated by centrifugation (9000×g, 30 min). The resulting fraction of 7S-globulins was washed with acidified water, centrifuged, dissolved in water (pH 7.5), dialyzed overnight, and lyophilized. The content of 7S- and 11S-proteins in the fractions was determined by gel densitometry as 90 and 93%, respectively. We used 10-15% gel colored by Coomassie Brilliant Blue. The gel was calibrated by 14-66 kDa MW markers (Pharmacia).

Preparation of Glycinin Hydrolysate. Glycinin was hydrolyzed by pepsin. The reaction was carried out at pH from 1.5 to 3.5 and from 25 to 60°C for 2-24 h in order to optimize the hydrolytic activity of the pepsin. The effective glycinin concentration was from 0.005 to 0.045 g/mL and was estimated for a pepsin concentration of 0.004 g/mL. The degree of hydrolysis was determined by measuring the absorption of trinitrobenzenesulfonic acid (TNBS) as before [18]. The content of protein-nitrogen in the glycinin hydrolysate was found using biuret [15].

Binding of Bile Acids with the Peptides. The in vitro interaction of bile acids was investigated by the literature method [19] analogous to that for the small intestine. Pure bile acids were evaluated separately. Each bile-acid solution contained phosphate buffer (31.25 mmol/mL, 0.1 M, pH 7.0). Samples were shaken for ~1 h at 37°C after adding bile-acid (4 mL) and pancreatin (5 mL) solutions, transferred quantitatively into plastic centrifuge tubes (50 mL), and centrifuged for 10 min at 26890×g. The supernatant was separated by Pasteur pipette into a second set of tubes, treated with phosphate buffer (5 mL) until the bile acids reddened, stirred, and centrifuged as described above. The supernatant was separated and combined. The amount of bile acids was determined as 1 mmol per 100 mg of blank and tested sample on a spectrophotometer at 530 nm.

Isolation of Peptides from Hydrolysate. Peptides from the pepsin hydrolysate were isolated, purified, and repurified by reversed-phase HPLC on a RP-HPLC (Jasco, Tokyo, Japan) with automatic gradient control, UV detector (220 nm), and Jasco integrator using a C_{18} semi-preparative column (Vydac 218TP510), CH_3CN mobile phase (0-70 min, 10-35%; 70-100 min, 35-100%), and 2 mL/min flow rate. Fractions containing peaks were collected manually and evaporated in vacuo automatically in a SpeedVac concentrator (Savant AS 260, Farmingdale, NY, USA).

Inhibition of HMG-CoA Reductase. The hypocholesterolemic effect of the pepsin-hydrolysate fractions was investigated in vitro and defined by percent inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA reductase). Fresh microsomes were isolated from rat liver (SD rats, 250 g, Hanlim Lab. Animal Co., Hwasong, Kyongki). HMG-CoA reductase activity was determined by the literature method [10]. The reaction mixture (1 mL) contained HMG-CoA (0.15 mM), NADP (2 mM), DTT (0.2 mM), and microsomal protein (1.0 mg) in triethanolamine (0.1 M) and EDTA (0.02 M) buffer (pH 7.4).

Identification of Amino-Acid Sequence. The amino-acid sequence of the fractions was established by Edman degradation [11] on an Applied Biosystems 491 Peptide Sequencer (Perkin—Elmer). The analysis was carried out according to the manufacturer's recommendations using Applied Biosystems chemical reagents.

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