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# In vitro binding of bile acids by lupin protein isolates and their hydrolysates

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**Abstract**

This study investigates the in vitro binding of bile acids by lupin, lupin protein isolates, and their hydrolysates compared to soybean products and cholestyramine. Sodium cholate, sodium deoxycholate, sodium chenodeoxycholate, sodium glycocholate and sodium taurocholate were individually tested and analyzed spectrophotometrically by enzymatic reaction. A degree of hydrolysis of up to 20% did not affect the bile-acid binding capacity. De-oiled lupin and its hydrolysate bound all the bile acids to a significantly greater extent than de-oiled soy and its hydrolysate. Acid-soluble protein isolate from lupin showed a greater bile-acid binding capacity than acid-insoluble protein isolate. The amount of bile acid bound by acid-soluble lupin protein isolate was sometimes greater than the amount of bile acid bound by cholestyramine, which is well known as a cholesterol-reducing agent. There was no selective binding of particular types of bile acids. It can be concluded from these results that acid-soluble protein isolate from lupin may have potential application as a cholesterol-reducing agent for hypercholesterolemic patients.


*Keywords:* *Lupinus albus*; Protein isolate; Bile-acid binding; Soybean; Hydrolysate

## 1. Introduction

Protein is an essential nutrient for growth and for regulating a wide range of body functions. Animal proteins, such as meat, cow's milk and eggs, and plant proteins, such as soybeans and nuts, are known to be good sources of protein. As animal protein sources often contain large amounts of saturated fat, the consumption of plant protein is recommended for reducing the risk of coronary heart disease. Various trials to replace a certain percentage of animal protein in food products with plant protein have been reported over recent decades and many different types of plant protein isolates have been produced. It was reported that the supplementation of plant protein isolate in model food products changed the product characteristics and nutritional values. Protein isolates are intended to be additives in food products for improving functional properties, such as the foaming/emulsifying capacity, gel formation, viscosity, texture and water-binding capacity. Protein isolates obtained from sesame seed (Lopez, Flores, Galvez, Quirasco, & Farres, 2003), defatted maize germ (Zayas & Lin, 1989) and peas (Dagorn-Scaviner, Gueguern, & Lefebvre, 1987), as well as wheat and soybean proteins (Boneldi & Zayas, 1995), have been added to a variety of products, usually as replacements for egg albumin.

Lupin seeds have a high protein content and nutritive value but they contain a large number of quinolizidine alkaloids which make the seeds bitter and potentially toxic. The Ministry of Agriculture, Fisheries and Food Department of Health UK (1996) mentioned that the oral LD50 of the lupin alkaloid extract in rats was 2300 mg/kg body weight. Since white lupin contained 0.003% of alkaloids (Wäsche, Müller, & Knauf, 2001), there is no risk of the toxicity of white lupin alkaloids for humans. Lupins contain a specific protein fraction, conglutin γ, which accounts for approximately 5% of the

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total protein content and has the exceptional characteristic of being a sulfur-rich protein; it contains amino acids that are scarce in other grain legumes. The interest in lupin proteins, and especially in lupin conglutin γ, is based on its unusual functional properties and nutritional benefits (Duranti, Sessa, Scarafoni, Bellini, & Dallocchio, 2000).

In order to utilize the lupin protein, we produced lupin protein isolates (Wäsche et al., 2001). In this work, a novel procedure has been proposed to maintain, two types of lupin protein isolates, one acid-soluble and the other a neutral pH-soluble fraction, in their native protein structures. Proteins were extracted from hexane-deoiled, flaked lupin kernels from low alkaloid-lupin genotypes. Although we reported an improvement in the physicochemical characteristics of protein isolates, the physiological effect, in terms of added value applications as functional ingredients of lupin protein isolate, was still unclear. Protein-enriched food fractions have the potential (on bile-acid binding) to lower cholesterol in blood (Sugano & Goto, 1990; Camire & Dougherty, 2003), and have effects on mineral bioavailability (Claye, Idouraine, & Weber, 1998), or ACE inhibition (Mullally, Meisel, & FitzGerald, 1997).

Hypercholesterolemia is an excessive level of cholesterol in the blood and this condition increases the risk of heart disease. Exercise, a reduced saturated fat intake and a higher dietary fibre intake are recommended for decreasing the blood cholesterol level. Eastwood and Hamilton (1968) reported that dietary fibres were responsible for binding bile acids. As bile acids are products of cholesterol decomposition in the liver, the effect of fibre/bile-acid binding on cholesterol lowering is based on the negative feedback of bile acids in the enterohepatic cycle (Pandolf & Clydesdale, 1992). Bile acids are secreted into the duodenum after conjugation with glycine or taurine, and they assist the digestion of fat by the formation of micelles. Bile-acid binding therefore also affects the fat digestion ratio. Bile-acid binding by dietary fibre is well reported; however, only a few reports are available on bile-acid binding by protein. Kahlon and Woodruff (2002) reported in vitro binding of bile acids by soy protein and some beans. Some reports have already mentioned the effect of protein for combating hypercholesterolemia (Anderson, Lovati, & Cook-Newell, 1995; Sirtori et al., 1998).

As a part of the work to determine the health benefits of food fractions, we have investigated bile-acid binding by lupin protein isolates and compared this to soybean and cholestyramine. Unlike dietary fibre, protein is not resistant to digestion. Some studies have found that protein hydrolysate has higher binding potential than the protein itself (Mullally et al., 1997; Pihlanto-Leppälä, Rokka, & Korhonen, 1998). In order to investigate this, protein, protein isolates and their hydrolysates were prepared and tested for bile-acid binding.

## 2. Materials and methods

### 2.1. Materials

White Lupin (*Lupinus albus*) seeds were obtained from Chile. Soybeans were obtained from the United States. In a dry milling step, the seed kernels and hulls were separated. The kernels were then flaked with a roll mill. The oil fraction was extracted from the flaked kernels, using hexane as the solvent. These de-oiled flakes were milled using a Retsch ZM-100 mill (Düsseldorf, Germany) to form a powder (<0.1 mm).

The lupin proteins were extracted from the de-oiled flakes using a two-stage process (Wäsche et al., 2001). In the first stage, the de-oiled flakes were mashed in cold water under acid conditions. The clarified acid extract was concentrated by cross-flow membrane filtration and was then spray-dried (this protein isolate was called Protein isolate F in the report of this work). In the second stage, the main storage protein fraction was extracted under neutral pH conditions and the soluble protein was collected and spray-dried (this protein isolate was called Protein isolate E).

Cholestyramine, sodium cholate, sodium deoxycholate, sodium chenodeoxycholate, sodium taurocholate, sodium glycocholate and bile-acid analysis kits were purchased from Sigma (St. Louis, MO). All the other reagents that were used for the experiments were analytical grade.

### 2.2. Methods

#### 2.2.1. Proximate analysis

The chemical compositions (dry matter content, protein content, oil and ash content) of de-oiled lupin flakes, soybean flakes and the processed lupin protein samples were determined in accordance with the LMBG method (LMBG, 1995).

#### 2.2.2. Digestion of samples

The sample digestion was done by the method of Pihlanto-Leppälä et al. (1998) and the method of van der Ven, Gruppen, de Bont, and Voragen (2002) with some modification. De-oiled lupin, lupin protein isolates and de-oiled soybean were digested with pepsin (enzyme/substrate ratio = 1:200) for 2 h at 37 °C at pH 2.0 and then heated at 80 °C for 20 min. For pepsin plus pancreatin digestion, the pH of the pepsin-digested samples was adjusted to pH 7.0 with NaOH before digestion with pancreatin (enzyme/substrate ratio = 1:200) for 3 h at 37 °C. The samples were then heated at 80 °C for 20 min. Digested samples were lyophilized and kept at 4 °C prior to use. The degree of hydrolysis was determined by the method of Nielsen, Petersen, and Dambmann (2001).

*2.2.3. Bile-acid binding assay*

The in vitro bile-acid binding procedure was a modification of that by Camire and Dougherty (2003) and Kahlon and Woodruff (2002). A buffer solution (0.1 M phosphate buffer at pH 7.0) was added to the de-oiled lupin, lupin protein isolate, de-oiled soybean and the digested samples to make a 10 mg/ml suspension. One hundred microlitres of a sample suspension (10 mg/ml) was transferred to a test tube and 900 μl of 2 mM bile-acid solution in 0.1 M phosphate buffer at pH 7.0 were added. As the bile-acid concentration in the human body is 1.5–7 mM (Calvert & Yeates, 1982), it was adjusted to that range.

After incubation at 37 °C for 2 h, each sample was centrifuged and the supernatant was transferred to a volumetric flask. A further 1 ml of 0.1 M sodium phosphate buffer at pH 7.0 was added to the sediment, mixed well and centrifuged. The supernatant was removed and combined with the earlier supernatant. This procedure was repeated and the supernatant was added to the existing supernatant in the volumetric flask. Aliquots of the pooled supernatant were frozen at −20 °C until analysis was carried out. The bile acids were analyzed spectrophotometrically at 530 nm using Sigma bile-acid analysis kit 450. The experimental values were determined from a standard curve obtained using tested bile-acid solutions. The individual substrate blanks were subtracted and the bile-acid concentrations were corrected for the mean recovery of bile acid (positive blank). Cholestyramine resin, a drug that binds bile acid and lowers cholesterol, was also evaluated for its ability to bind bile acid. All analyses were at least performed in triplicate.

*2.2.4. Statistical analysis*

Results are presented as mean values ± s.e.m. ($n = 3$ to 8). ANOVA was used to calculate significant differences.

Table 1
Chemical composition of de-oiled lupin, lupin protein isolates and de-oiled soybean

| Sample | Dry matter (%) | Protein (N × 6.25) (%) | Oil (%) | Ash (%) |
|---|---|---|---|---|
| De-oiled lupin | 89.69 | 56.8 | 1.95 | 3.82 |
| Protein isolate F | 93.53 | 91.44 | 0.24 | 0.30 |
| Protein isolate E | 96.17 | 100.62 | 1.16 | 0.15 |
| De-oiled soybean | 90.00 | 61.0 | 2.00 | 6.00 |

Table 2
Degree of hydrolysis of de-oiled lupin, lupin protein isolates and de-oiled soybean

| Sample | Pepsin | Pepsin + pancreatin |
|---|---|---|
| De-oiled lupin | 2.88 | 13.2 |
| Protein isolate F | 3.26 | 18.2 |
| Protein isolate E | 0.36 | 11.5 |
| De-oiled soybean | 3.34 | 5.66 |

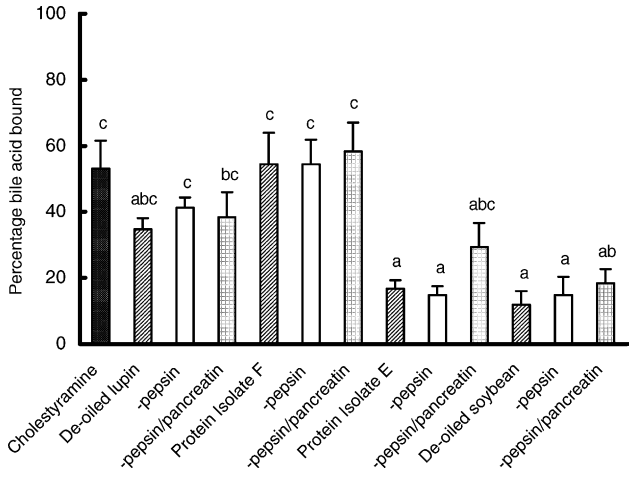


Fig. 1. Sodium cholate binding by lupin, lupin protein isolates, soybean and cholestyramine. Different letters indicate significant differences ($p < 0.05$).

## 3. Results and discussion

We tested five bile acids for in vitro binding by de-oiled lupin, lupin protein isolates, de-oiled soybean and their hydrolysates. The results of proximate analyses are shown in Table 1. Lupin, lupin protein isolates and soybeans had dry matter contents of 89.7–96.2%.. Protein isolate F had the lowest concentration of oil (0.24%). Protein isolates E and F had higher protein contents, expressed as nitrogen (N) × 6.25, at 91–100%. This showed that the protein isolate production process was efficient. As the proximate analyses were carried out by the LMGB method, the protein content was slightly higher than that calculated using the AOAC method. As previously reported (Wäsche et al., 2001), alkaloid content of lupin was 0.003%, and by protein isolation, it became less than 0.001%. This decrease indicate that removal of alkaloid was efficient in protein isolation. The degree of hydrolysis of the samples is shown in Table 2. Protein isolates were hydrolyzed to a greater degree than lupin and soybean samples, which included dietary fibres.

The sodium cholate binding by lupin, lupin protein isolates and soybean is shown in Fig. 1. Sodium cholate was bound by de-oiled lupin and its hydrolysate to a degree of 34.7–41.3%, by protein isolate E and its hydrolysate 14.8–29.4%, by protein isolate F and its hydrolysate 54.4–58.3% and by cholestyramine to 53.1%. When degree of hydrolysis was increased, the sodium cholate binding capacity was not significantly affected by hydrolysis. Lupin protein isolate F and cholestyramine showed significantly higher sodium cholate

binding than de-oiled soybeans and its hydrolysate (11.9–18.5%). Story and Krichevsky (1976) tested sodium cholate binding by cholestyramine and alfalfa at a cholate concentration of 10 mM. They reported that 60.7% of cholate was bound by cholestyramine and 19.9% of cholate was bound by alfalfa. Camire, Zhao, and Violette (1993) and Camire and Dougherty (2003) reported cholate binding by cholestyramine, three types of raisins, wheat bran and various types of potato peels at a cholate concentration of 12.5 mM. Their work indicated cholate binding of 75%, 15–20%, 10% and 1.9–8.1%, respectively. Raisins, wheat bran and potato peels, de-oiled lupin and lupin protein isolate F showed higher cholate binding capacity than alfalfa, while soybean showed similar cholate binding to that in the cited references (Camire & Dougherty, 2003; Camire et al., 1993).

As shown in Fig. 2, sodium chenodeoxycholate was bound by de-oiled lupin/hydrolysate to a degree of 39.2–45.6%, by protein isolate E/hydrolysate 14.0–20.9%, by protein isolate F/hydrolysate 55.8–71.4%, by de-oiled soybean/hydrolysate 14.3–17.6% and by cholestyramine 60.8%. The sodium chenodeoxycholate binding capacity was also not significantly affected by hydrolysis. Soybeans/hydrolysate showed significantly lower chenodeoxycholate binding than cholestyramine and protein isolate F/hydrolysate. Chenodeoxycholate was reported to be bound to a degree of 86.5% by cholestyramine and 24.8% by alfalfa (Story & Krichevsky, 1976). Protein isolate E showed slightly lower chenodeoxycholate binding than alfalfa, while protein isolate F exhibited higher chenodeoxycholate binding, nearly the same as for cholestyramine.

Fig. 3 shows that sodium deoxycholate was bound by de-oiled lupin/hydrolysate to a degree of 34.3–57.7%, by protein isolate E/hydrolysate 12.8–19.5%, by protein isolate F/hydrolysate 58.4–69.5%, by de-oiled soybean/hydrolysate 12.6–15.0% and by cholestyramine 66.4%. The sodium deoxycholate binding capacity was also not significantly affected by hydrolysis. The percentage of deoxycholate bound by the tested samples was slightly higher than was cholate (no significant difference). Cholestyramine has been reported to bind deoxycholate under various in vitro conditions to a degree of 92.5% (Story & Krichevsky, 1976), 85% (Camire & Dougherty, 2003) and 99.9% (Camire et al., 1993). Compared to these values, our results showed lower deoxycholate binding by cholestyramine. Alfalfa (Story & Krichevsky, 1976), raisins, wheat bran (Camire & Dougherty, 2003) and potato peels (Camire et al., 1993) were reported to bind deoxycholate to degree of 10.8%, 5–10%, 15% and 10.6–18.9%, respectively. Compared to these samples, our lupin protein isolate F showed a much higher deoxycholate binding capacity. Cholic acid and chenodeoxycholic acid are primary bile acids which are produced by the body. In contrast, deoxycholic acid is a secondary bile acid which is produced by microorganisms living in the gastrointestinal tract. The high concentration of secondary bile acid may cause intestinal inflammation. It is hence desired to have greater binding of deoxycholic acid than cholic acid.

The result of the sodium glycocholate binding tests is shown in Fig. 4. Sodium glycocholate was bound by de-oiled lupin/hydrolysate to a degree of 42.7–57.5%, by protein isolate E/hydrolysate 10.5–28.1%, by protein isolate F/hydrolysate 63.9–68.0%, by de-oiled soybean/hydrolysate 0.2–8.5% and by cholestyramine 25.0%. Soybean and its hydrolysates had significantly lower binding capacities than the other samples that were tested. Protein isolate F showed significantly higher glycocholate binding than cholestyramine and protein isolate E. The binding capacity of protein isolate F was

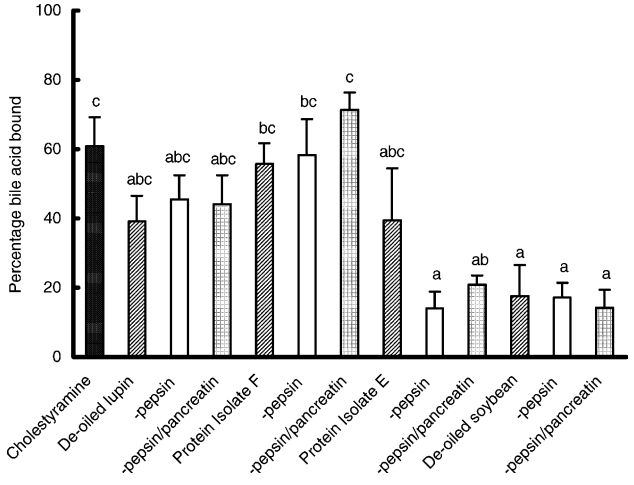


Fig. 2. Sodium chenodeoxycholate binding by lupin, lupin protein isolates, soybean and cholestyramine. Different letters indicate significant differences ($p < 0.05$).

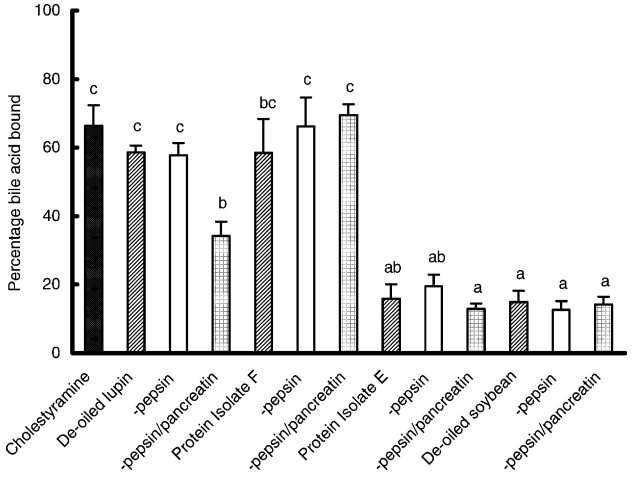


Fig. 3. Sodium deoxycholate binding by lupin, lupin protein isolates, soybean and cholestyramine. Different letters indicate significant differences ($p < 0.05$).

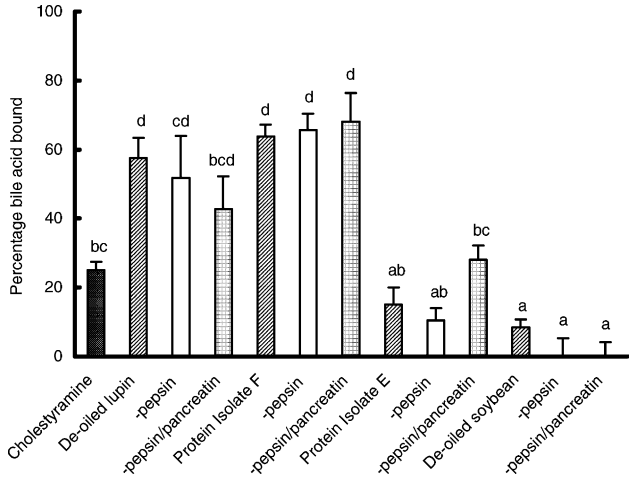


Fig. 4. Sodium glycocholate binding by lupin, lupin protein isolates, soybean and cholestyramine. Different letters indicate significant differences ($p < 0.05$).

not affected by hydrolysis. Both pepsin hydrolysate F and pepsin–pancreatin hydrolysate F also exhibited significantly higher glycocholate binding than cholestyramine and protein isolate E/hydrolysate. Cholestyramine has been reported to bind glycocholate under various conditions to a degree of 74.2% (Story & Krichevsky, 1976) and 100% (Camire & Dougherty, 2003). Alfalfa and wheat bran were shown to have a glycocholate binding capacity of 11.5% (Story & Krichevsky, 1976) and 100% (Camire & Dougherty, 2003). Lupin protein isolate F showed higher glycocholate binding than alfalfa.

As shown in Fig. 5, sodium taurocholate was bound by de-oiled lupin/hydrolysate to a degree of 36.4–40.3%, by protein isolate E/hydrolysate to a degree of 5.76–13.1%, by protein isolate F/hydrolysate to a degree of

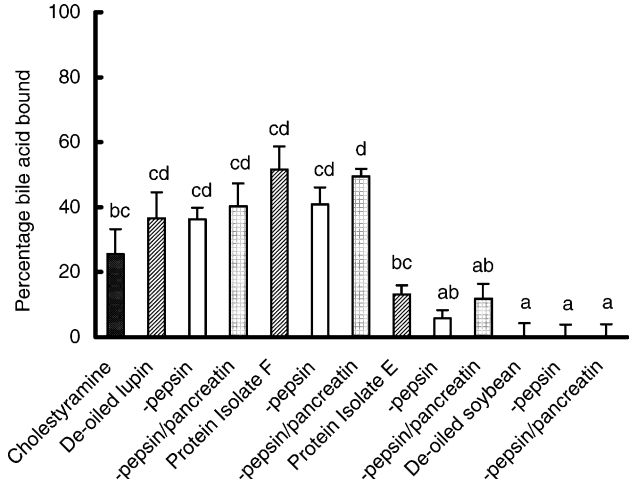


Fig. 5. Sodium taurocholate binding by lupin, lupin protein isolates, soybean and cholestyramine. Different letters indicate significant differences ($p < 0.05$).

40.9–51.6% and by cholestyramine to a degree of 25.7%. De-oiled soybean/hydrolysate did not bind sodium taurocholate. The sodium taurocholate binding capacity was also not significantly affected by hydrolysis. Cholestyramine showed significantly lower taurocholate binding than pepsin–pancreatin-digested protein isolate F. Compared to other bile acids, the taurocholate binding by cholestyramine was lower. Cholestyramine was reported to bind taurocholate to a degree of 80.7% (Story & Krichevsky, 1976) and 75% (Camire & Dougherty, 2003), however, cholestyramine showed lower glycocholate and taurocholate binding than in other reports above mentioned. This was supposed to be a result of depending on the range of experimental conditions. Alfalfa, wheat bran and rhubarb were reported to bind taurocholate to a degree of 6.9% (Story & Krichevsky, 1976), 9–10% (Camire & Dougherty, 2003; Goel et al., 1998) and 24.6% (Goel et al., 1998). In our study, lupin protein isolate F showed higher taurocholate binding than alfalfa, wheat bran and rhubarb.

For all five of the bile acids under test, de-oiled lupin, lupin protein isolate F and cholestyramine showed higher bile-acid binding than protein isolate E and de-oiled soybeans. Iwami, Sakakibara, and Ibuki (1986) and Sugano and Goto (1990) reported that hydrophobic undigested fractions of soy protein have been shown to lower cholesterol and bind bile acids to an even greater extent than soy protein. However, in our study, the bile-acid binding capacity was not affected by hydrolysis (digestion) up to a degree of hydrolysis of 20%. Kahlon and Woodruff (2002) tested bile-acid binding with bile acid mixtures at a total concentration at 288 μM. In order to make a comparison with our results, we have converted their data to "% bound" values. Cholestyramine bound 95% of the bile acid mixture. Compared to cholestyramine, soybean showed approximately 15% of the bile-acid binding capacity (for the total bile acid mixture, 14.3% of the bile acid mixture was bound by soybean). In our results, soybean showed 19.0–34.8% binding (simple bile acids) and 0–34.0% bile acids binding (conjugated bile acids) relative to cholestyramine, depending on the type of bile acids. Duranti et al. (2000) reported a specific fraction of lupin protein, conglutin γ, and how this unusual protein may affect the bile-acid binding or may induce higher bile-acid binding capacity. Further research with purified conglutin γ is also required with analysis of the conglutin γ content in each protein isolate fraction in order to verify the physiological function of lupin protein.

In this study, we tested each bile acid separately, although physiological conditions usually involve bile acid mixtures. It is planned to undertake further studies with bile acid mixtures, using HPLC or capillary electrophoresis to characterize the individual bile acids. Such studies will clarify the functions and potential applications of protein isolates. The present work has identified

a useful property of lupin protein isolate, in particular protein isolate F, to bind bile acids to nearly the same extent as cholestyramine. The results suggest that acid-soluble protein isolate from lupin may have potential applications as an agent for reducing cholesterol in hypercholesterolemic patients.

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