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Amino acid composition and *in vitro* digestibility of protein isolates from *Silybum marianum*

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Abstract

To assess the protein quality and the nutritive value of *Silybum marianum* protein (SMP), amino acid composition, differential scanning calorimetry (DSC) and the *in vitro* digestibility were determined and compared with those of soy protein isolate (SPI). The results showed that SMP had an excellent balance of all essential amino acids, with a relatively high level of glutamic acid, arginine, leucine and valine. Except lysine, the essential amino acids of SMP met the suggested requirements of FAO/WHO for 2-5 year old infants. The proportion of essential amino acids to the total amino acids (E/T) for SMP was higher than that of SPI. All the estimated nutritional quality parameters based on amino acid composition showed that SMP had good nutritional quality. SMP had a single denaturation temperature (97.8°C). In an *in vitro* digestion model, SMP was easily digested by pepsin plus trypsin. In contrast, SMP was much more easily digested by trypsin than SPI. After pepsin plus trypsin digestion, SMP and SPI were all digested to release oligo-peptides and amino acids. These results about SMP are important for its potential application as functional food ingredients.

Key words: *Silybum marianum* protein, amino acid composition, DSC, *in vitro* digestibility.

Introduction

Silybum marianum is an annual or biennial plant. It is known as lady's thistle, holly thistle, marian thistle, and belongs to family Asteraceae. *Silybum marianum* is native of southern Europe, mainly the Mediterranean regions, indigenous to North Africa, Asia Minor and Southern Russian Federations. *Silybum marianum* is now naturalized throughout Europe, in North and South America and Australia [1]. In China, it mainly distributes in Qinghai,Shaanxi, Jilin, Jiangsu and Guangdong provinces [2].Various preparations of the plant, especially the fruits, have been used medicinally to treat liver disorders for over 2000 years [3]. The main active constituents of this plant are flavonolignans collectively known as silymarin. Now there is a growing interest in its anticancer as well as chemopreventive, hypocholesterolemic, cardioprotective, neuroactive and neuroprotective activities [4-9].

Silybum marianum seed have two parts of shell and kernel. Silymarin is mainly in the seed shell and seed kernel contains mainly protein and oil [10]. The oil contains a relatively high content of vitamin E and a great quantity of the unsaturated fatty acids such as linoleic (C18:2) and oleic acid (C18:1) [11-13]. The protein (mainly albumin) in seed kernel is also very nutritional in essential amino acids, and the processing properties of the protein were excellent. The solubility of the protein was better and its foaming capacity and foam stability, emulsification capacity and stability were remarkably superior to that of the SPI [14, 15]. Thus, the protein from seed kernel has good potential to be applied as a valuable source of protein nutrition. However, there were few reports exclusively aimed at evaluating the nutritional quality and *in vitro* digestibility of SMP.

Therefore, it is necessary to evaluate the nutritional quality and physico-chemical properties of SMP. The main objective of this study was to evaluate amino acid composition, DSC and *in vitro* digestibility of SMP comparing to SPI.

Materials and Methods

Materials: SMP was prepared in our laboratory by alkali extraction and acid precipitation method [16]. A commercial SPI was purchased from Sanwei Soy Protein Co., Ltd (Linyi, China). Pepsin and trypsin were purchased from Sigma (St. Louis, USA). Low molecular weight protein markers were purchased from Takara Biotech. Co., Ltd. (Dalian, China). All Other chemicals used were of analytical grade.

Chemical analysis: The proximate compositions (including protein, fat, ash and moisture) of SMP and SPI were determined according to AOAC procedures [17].

Amino acid composition analyses: For the determination of the amino acids, the sample of protein (100 mg) was subjected to acid hydrolysis with 5 ml of 6 M HCl under nitrogen atmosphere for 24 h at 110°C. The hydrolyzate was washed into a 50 ml volumetric flask and made up to the mark with distilled water. The amino acids were subjected to RP-HPLC analysis (Agilent 1100) after precolumn derivatization with o-phthaldialdehyde (OPA) [18]. Amino acid composition was reported as g of amino acid/100 g of protein.

Parameters of nutritional quality: The nutritional parameters of SMP and SPI were calculated using their amino acid composition including:

1) The proportion of essential amino acids to the total amino acids

of the protein (E/T) [19].

$$E/T\% = \frac{Ile+Leu+Lys+Met+Cys+Phe+Tyr+Thr+Val+His}{Ala+Asp+Arg+Gly+Glu+Ile+Leu+Lys+Met+Cys+Phe+Tyr+Pro+Ser+Thr+Val+His} \times 100$$

2) Amino acid score (AAS) [19]:

AAS=(mg of amino acid per g of test protein/mg of amino acid per g of FAO/WHO standard pattern)×100.

3) Predicted protein efficiency ratio (PER) values.
The predicted PER values was estimated by three equations [19], as given below:

PER (1) = -0.684 + 0.456 (Leu) - 0.047 (Pro)

PER (2) = -0.468 + 0.454 (Leu) - 0.105 (Tyr)

PER (3)= -1.816 + 0.435(Met) + 0.780(Leu) + 0.211(His) - 0.944 (Tyr)

4) Protein digestibility-corrected amino acid score (PDCAAS).
PDCAAS was calculated from the amino acid score and *in vitro* digestibility as described by Abdul-Hamid *et al.*[20].

PDCAAS= the lowest amino acid score × protein digestibility

5) *In vitro* protein digestibility(IVPD).
In vitro digestion of samples was performed with pepsin plus trypsin as described according to the method of Njingtang *et al.* [21] and Nunes *et al.* [22], with some modifications. Briefly, protein sample was dispersed in 0.1 M HCl (pH 1.5) (1%, w/v), and incubated in a water bath at 37°C for 3-5 min. An aliquot of pepsin powder (1:8000 U/gprot) was added and mixed, and the mixture was incubated at 37°C for 120 min. The pH of the mixture was adjusted to 7.0 with 1.0 M NaOH to stop the digestion reaction. The neutralised pepsin-digested mixture was mixed with trypsin powder (1:8000 U/gprot) to initiate further digestion for another 120 min. The digested sample was mixed with an equal volume of 10% TCA. Sample was centrifuged (8,000 g) for 20 min. The content of TCA soluble nitrogen of the supernatant was determined by micro-Kjeldahl nitrogen analysis.

In vitro digestibility was reported as the percentage of soluble nitrogen, as given below:

IVPD = mg of NPN in supernatant / mg of total N content of undigested sample × 100

Differential scanning calorimetry (DSC): The thermal denaturation of the protein was examined with DSC 200F3 (Netzsch, Germany). Lyophilized sample (1 mg) was directly weighed into the aluminium pans and 10 µl of 0.01M pH 7.5 phosphate buffer was added. An empty pan was used as a reference. Scanning was done at 20~150°C at a heating rate of 10°C/min. Thermal denaturation temperature (T_d) and denaturation entalphy (ΔH) were calculated from thermograms.

Sequential in vitro protein digestion procedure: The *in vitro* digestibility of SMP and SPI were evaluated using sequential pepsin and trypsin digestion model according to the method of Nunes *et al.* [22] and Wang *et al.* [23], with some modifications. For pepsin digestion, in a 100 ml centrifuge tube, 0.8 g of protein material was suspended in 75 ml of 0.1 M HCl (pH 1.5), and mixed with pepsin power in 0.5 ml of 0.1 M HCl. The mixture of protein and pepsin was incubated at 37°C for 120 min, under gently shaking condition. After that, the pepsin-digested hydrolysate was neutralized with 1.0 M phosphate buffer (pH 8.0), followed by the addition of appropriate trypsin. This mixture (of pepsin-digested hydrolysate and trypsin) was incubated at 37°C for another 120 min.

For SDS-PAGE analysis of digestion process, 200 µl aliquots of the protein and enzyme mixtures were taken at specific periods of incubation time (0-120 min), during pepsin and subsequent trypsin digestion. These mixtures were directly mixed with the same volume of the sample buffer (in order to inactivate the enzyme and at the same time prepare the samples suitable for SDS-PAGE analysis). The SDS-PAGE was performed as follows. SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli [24] using 15% separating gel and 3% stacking gel. The mixtures were heated for 5 min and centrifuged (10,000 g, 10 min) before electrophoresis. For each sample, 10 µl was applied to each lane. After the electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

Statistical analysis: An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

Results and Discussion

Proximate chemical composition of SMP and SPI: The protein, moisture, fat and ash contents of SMP and SPI are shown in Table 1. The obtained SMP was mainly composed of protein (87.52%), moisture (4.2%), fat (0.68%), ash (2.28%) and other components (e.g., carbohydrate). The contents of these constituents for SMP were similar to that of SPI. By comparison, SMP had lower protein content. SMP and SPI had similar low fat and ash contents.

Table 1. Proximate chemical composition of SMP and SPI (g/100 g).

	Protein	Moisture	Fat	Ash
SMP	87.52±0.23	4.20±0.12	0.68±0.02	2.28±0.02
SPI	93.18±0.52	3.63±0.18	0.48±0.01	2.80±0.01

Means±SD; values are means of three determinations.

Analysis of amino acid composition: SMP and SPI were analyzed for amino acid composition and the results are presented in Table 2. In general, like SPI, SMP had a well-balanced amino acid composition. Glutamic acid, arginine, leucine, and glycine were all abundant in the protein. In addition, the contents of aspartic acid and valine were high in SMP. Although the sulfur-containing amino acids (Met + Cys) might be to some extent destroyed by the HCl-hydrolysis method used in this study, their contents in SMP were higher than that of SPI. Infants have very critical nutritional requirements due to rapid growth and immaturity of gastrointestinal function, and nine amino acids have been identified to be essential for infants: Thr, Val, Leu, Ile, Lys, Trp, Phe, Met and His. Arg and Cys are also essential for low birth weight infants. In comparison, the essential amino acids Lys and Phe of SMP were to a various extent lower than that of SPI, however, the others were similar or higher. According to the FAO/WHO suggested requirements for 2-5 year old infants, only lysine in SMP is limiting amino acid. Except this amino acid, other essential amino acids are sufficient for the FAO/WHO suggested requirements for 2-5 year old infants. SMP was prepared by alkaline water extraction and isoelectric precipitation from *Silybum marianum* cake, so the lower contents of cystine, threonine and lysine may have been caused by alkaline

Table 2. Amino acid composition of SMP and SPI (g/100 g of protein).

Amino acids	SMP	SPI	FAO/WHO for preschool child
Essential amino acids			
Ile	5.21	4.81	2.8
Leu	7.98	7.18	6.6
Lys	4.83	6.05	5.8
Met	1.86	2.05	
Met+Cys	2.84	2.44	2.5
Phe	4.81	5.24	
Phe+Tyr	9.02	10	6.3
Thr	4.45	3.97	3.4
Val	6.21	4.03	3.5
His	2.89	3.18	1.9
Nonessential amino acids			
Asp	8.79	10.62	
Glu	18.27	19.14	
Arg	7.51	8.81	
Ser	5.28	5.04	
Gly	6.91	4.76	
Pro	4.58	6.45	
Ala	5.18	3.51	
Cys	0.98	0.39	
Tyr	4.21	4.76	

processing, which can cause destruction of cystine, threonine, arginine, serine and lysine [25]. Therefore, effective controlling of the pH value is very important during protein isolate preparation.

Classification of amino acids in different groups according to chemical properties are shown in Table 3.The content of hydrophobic amino acids in SMP (35.86%) was most abundant compared to the other groups (acidic, basic and uncharged polar). The second were the acidic ones with levels around 27.07%, directly followed by the uncharged polar (21.83%) and basic amino acids (15.22%). The relative ratio of acidic and basic amino acids would determine the net charge on the surface of protein, since charged residues are mostly located on the surface of the protein molecule. The content of acidic amino acids was higher than that of basic, thus the isoelectric point of SMP was lower [16]. The sulfur-containing amino acids in SMP were higher than that of HPI and SPI, but the aromatic amino acids in SMP were just the reverse [26]. Moreover, the contents of branched chain amino acid in SMP (19.41%) were higher than that of SPI (16.01%).

Table 3. Distribution of amino acid classified according to similar chemical properties in SMP and SPI (g/100g of protein).

Group	SMP	SPI
Hydrophobic (nonpolar)[a]	35.86	33.26
Uncharged polar[b]	21.83	18.90
Basic[c]	15.22	18.04
Acidic[d]	27.07	29.78
Sulfur-containing[e]	2.84	2.44
Aromatic[f]	9.02	9.99
Branched chain amino acid[g]	19.41	16.01

[a] Ala,Val,Leu,Pro,Met,Phe and Ile. [b] Ser,Thr,Cys,Gly andTyr. [c] Lys,Arg, and His. [d] Asp and Glu. [e] Cys and Met. [f] Phe,and Tyr. [g] Val,Leu and Ile.

Estimated nutritional quality based on amino acid composition: Protein is one of the most important nutrients in the human diet. Both the amount and quality of protein provided by daily food are important. The protein quality, also known as the nutritional or nutritive value of a food, mainly depends on its amino acid content and the physiological utilization of specific amino acids after digestion, absorption, and so on. Because direct assessment of protein nutritional value in human subjects is impractical for regulatory purposes, the methods based on *in vitro* (chemical) and animal bioassays for assessment of protein quality have been developed. In this paper, we use the amino acid data as a basis for estimation of nutritional quality of SMP. The ratio of essential to total amino acids, amino acid score, limiting amino acids, PER, PDCAAS of SMP and SPI are shown in Tables 4 and 5.

Table 4. Amino acid scores (AAS) of SMP and SPI compared with the FAO/WHO pattern.

Amino acid	FAO/WHO pattern (g/100 g)	SMP	SPI
Ile	2.8	186	171
Leu	6.6	121	109
Lys	5.8	83	104
Met+Cys	2.5	114	98
Phe+Tyr	6.3	143	159
Thr	3.4	131	117
Val	3.5	177	115
His	1.9	152	167

Table 5. Nutritional evaluation of SMP and SPI.

Parameters	SMP	SPI
E/T/%	43.43	41.64
Amino acid score/%	83	98
First limiting amino acids/%	Lys	Met+Cys
Second limiting amino acids/%	Met+Cys	Lys
PER1	2.74	2.28
PER2	2.71	2.29
PER3	1.85	0.85
IVPD /%	86.92	87.02
PDCAAS	0.72	0.85

SMP had a higher ratio of essential to total amino acids (43.43%) than the pattern recommended by FAO/WHO (at least 36%) and SPI (41.64%) in Table 5. The amino acid scores of SMP were all more than 100 except for the cereal limiting amino acid lysine (Table 4). SPI had a higher AAS of 98 than SMP (83). In contrast, SMP exhibited high scores for isoleucine (186) and valine (177). Lysine was the first limiting amino acids and sulfur containing amino acids were the second limiting amino acid, SPI was just the reverse. Predicted PER values all exceed 2.00, which describes a high quality protein [18]. The PER1 and PER2 values of SMP all exceed 2.70, but the PER3 values was 1.85, higher than that of SPI (0.85). The digestibility of proteins is a major factor in their quality assessment. *In vitro* protein digestibility (IVPD) of SMP and SPI was evaluated. Like SPI, *in vitro* protein digestibility of SMP (86.92%) was higher than that of legumes proteins (50-70%) [27]. AAS is a measure of the actual amounts of individual amino acids in a food, or in the diet relative to the need for this amino acid. This ratio does not evaluate whether the protein is digestible or not. So the FAO/WHO has adopted a new scale called the protein digestibility-corrected amino acid score (PDCAAS). While not perfect, it is much better and more accurate in relation to the true needs of humans and the scoring of food. The PDCAAS was directly related to the *in vitro* digestibility. The PDCAAS of SMP was 0.72, lower than that of SPI (0.85).

In short, compared with earlier observations, the SMP amino acid profile gives a good balance of total essential amino acids, limited only in lysine, having a high nutritional quality. The deficiency of lysine, methionine and cysteine could be supplemented by other proteins as milk proteins.

Differential scanning calorimetry (DSC): DSC is a rapid, easy and capable technique for supplying both thermodynamic (heat capacity, enthalpy and entropy) and kinetic data (reaction rate and activation energy) on protein denaturation, and has been used extensively in various food systems. The DSC thermograms of SMP is presented in Fig.1. In the thermal transition of SMP, a prominent endothermic peak was observed. The denaturation temperature (T_d) of the protein was about 97.8°C, higher than that of SPI (84.2 °C) [28]. The endothermic peak in the DSC thermograms of proteins is usually related to disruption of hydrogen bonds, especially those maintaining the integrity of tertiary structure of the proteins. Thus, the endothermic peak for SMP may be due to strong hydrogen bond interactions maintaining the tertiary conformation. The enthalpy change (ΔH) of the endothermic peak of SMP was 1120 J/g, significantly higher than that of SPI [28]. The ΔH reflects the proportion of undenatured protein in a sample, or extent of ordered structure. Thus, this data suggests that the extent of ordered structure in SMP was higher than that in SPI. Therefore, the results suggest that SMP has high thermal stability, not easy denaturation.

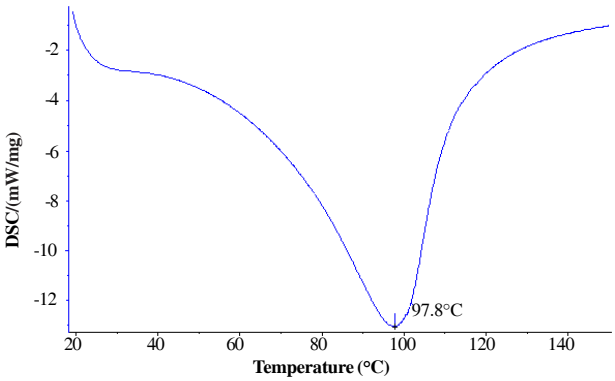

Figure 1. Differential scanning calorimetry thermograms of SMP.

Sequential in vitro protein digestibility: The *in vitro* digestibility of SMP and SPI was evaluated using the sequential pepsin and trypsin digestion model, by reducing SDS-PAGE as shown in Figs 2 and 3. During the pepsin digestion, the protein constituents of SMP were rapidly digested by pepsin within about 1 min, to release oligo-peptides with molecular weight (MW) less than 14.0 kDa in reducing SDS-PAGE profile (Fig. 2). In a similar way, SPI was digested by pepsin. Upon further incubation with pepsin, it could be distinctly observed that the MW distribution of the oligo-peptides decreased with increasing the incubation time from 1 to 120 min. After the pepsin-digested hydrolysate was adjusted to pH 8.0, the addition of trypsin led to further decline in the MW distribution of the oligo-peptides (Fig. 3). The pepsin and trypsin digestion pattern of SMP was similar to that of SPI. In contrast, the subunits of soy β-conglycinin were much less prone to pepsin digestion. SMP was easily digested by trypsin. This difference may be attributed to the difference in protein stability of these subunits in acid medium (at about pH 2.0). The results suggest that SMP is a good source of much more digestible protein as compared to SPI, which is highly suitable for the human consumption.

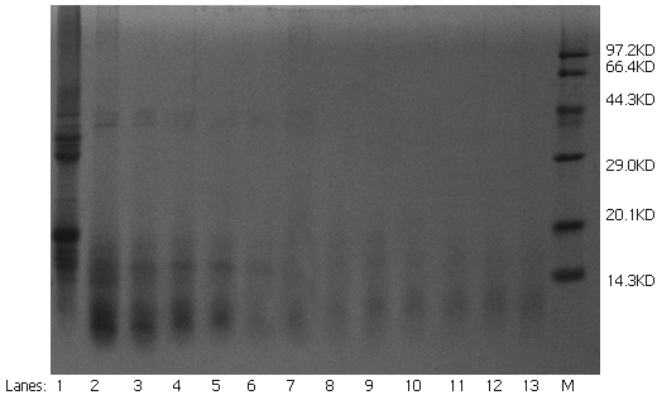

Figure 2. Reducing SDS-PAGE profiles for SMP digested with sequential pepsin and trypsin. M, standard protein. Lanes 1-7, SMP digested by pepsin for 0, 1, 5, 10, 30, 60 and 120 min, respectively; Lanes 8-13, the SMP pepsin-hydrolysate further digested by trypsin for 1, 5, 10,30, 60 and 120 min.

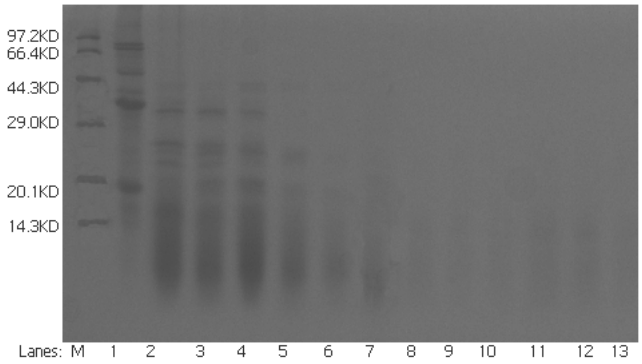

Figure 3. Reducing SDS-PAGE profiles for SPI digested with sequential pepsin and trypsin. M, standard protein. Lanes 1-7, SPI digested by pepsin for 0, 1, 5, 10, 30, 60 and 120 min, respectively; Lanes 8-13, the SPI pepsin-hydrolysate further digested by trypsin for 1, 5, 10,30, 60 and 120 min.

Conclusions

The amino acid composition and *in vitro* digestibility of SMP was investigated in this paper. SMP showed an excellent balance of all essential amino acids and had a good nutritional quality. According to the differential scanning calorimetry, SMP possessed higher thermal stability than SPI. The measure of the sequential *in vitro* pepsin digestibility by SDS-PAGE showed that SMP was more easily digested than SPI. The results could be useful for providing knowledge about the properties of SMP, thus facilitating the utilisation of this protein in nutrition and health food manufacture. Ultimately, SMP is currently underutilized and it is definitely worth attention as a source of health-promoting component for foods or might be as multifunctional food ingredients for food manufacture.

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