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Jojoba Seed Meal Proteins Associated with Proteolytic and Protease Inhibitory Activities

MADAN K. SHRESTHA,[†] IRENA PERI,[†,‡] PATRICIA SMIRNOFF,[‡] YEHUDITH BIRK,[‡] AND AVI GOLAN-GOLDHIRSH*,[†]

Desert Plant Biotechnology Laboratory, Albert Katz Department of Dryland Biotechnologies, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede Boker Campus 84990, Israel, and Institute of Biochemistry, Food Science & Nutrition, The Hebrew University of Jerusalem, Rehovot 76100, Israel

The jojoba, *Simmondsia chinensis,* is a characteristic desert plant native to the Sonoran desert. The jojoba meal after oil extraction is rich in protein. The major jojoba proteins were albumins (79%) and globulins (21%), which have similar amino acid compositions and also showed a labile thrombin-inhibitory activity. SDS−PAGE showed two major proteins at 50 kDa and 25 kDa both in the albumins and in the globulins. The 25 kDa protein has trypsin- and chymotrypsin-inhibitory activities. In vitro digestibility of the globulins and albumins resembled that of casein and soybean protein concentrates and was increased after heat treatment. The increased digestibility achieved by boiling may be attributed to inactivation of the protease inhibitors and denaturation of proteins.

KEYWORDS: Jojoba meal protein; *Simmondsia chinensis*; solubility; protease inhibitory activity; digestibility

INTRODUCTION

Jojoba (*Simmondsia chinensis* (Link) Schneider) is a shrub indigenous to the Sonoran deserts of Arizona, California, and Mexico. The jojoba was introduced as a cultivated crop for the commercially useful liquid wax in its seeds. The meal remaining after oil extraction is rich in protein (*1*). The use of the meal proteins for food and feed is limited because it contains antinutritional factors such as simmondsin and its derivatives (*2*), heat labile trypsin inhibitors (*3*), and condensed tannins with antitryptic activity (*4*). Research has already been carried out to remove the antinutritional factors in order to utilize the jojoba proteins for animal consumption (*1, 5*−*9*). Jojoba proteins were fractionated according to solubility by Cardoso and Price (*10*). Abbott et al. (*1*) reported a process for preparation of a water-soluble protein isolate. The presence of several endopeptidases in jojoba seed was noted by Samac and Storey (*3*), and further fractionation of these enzymes was performed by Wolf and Storey (*11*). Recently it was suggested that a trypsin inhibitory activity might be responsible for growth retardation in rats fed jojoba proteins (*9*), but a more detailed analysis of jojoba meal proteins and their characterization is lacking. In addition, there is increasing interest in recovering the jojoba meal proteins for industrial, cosmetic, and food uses, as well as to avoid disposal problems and environmental hazards after oil extraction. In view of the recent interest in plant protease inhibitors as built-in defense mechanisms against stored-product insect proteases (*12*) and as possible cancer chemopreventive agents, the jojoba meal proteins offer a source for the biotechnological uses of proteases and protease inhibitors.

In this paper we describe the isolation of major jojoba meal proteins, their in vitro digestibility, and the identification of serine protease inhibitory activity of a major 25 kDa jojoba meal protein, Joj25.

MATERIALS AND METHODS

Plant Material. Jojoba (*Simmondsia chinensis* (Link) Schneider) press-defatted meal was obtained by an industrial process (kindly provided by "Jojoba Israel", Kibbutz Chatzerim).

Fractionation of Meal Proteins. The meal proteins were extracted according to solubility by two methods (A, B) shown in **Figure 1**. In method A the starting material was used as obtained from the factory and in method B the meal was further defatted by solvent extraction as detailed in **Figure 1B**. Fractionation of the meal proteins was performed by submitting the albumins or globulins obtained by method B to column chromatography on Q-sepharose (Pharmacia Biotech Ab, Uppsala, Sweden). The proteins were eluted from the column by a stepwise gradient of 50 mM NaCl followed by 100 mM NaCl, and 200 mM salt, in 20 mM Tris/HCl buffer, pH 8.0. The eluate was monitored spectrophotometrically at 280 nm. Pooled protein fractions were dialyzed and lyophilized. The fraction containing Joj25 peak was applied to HPLC-Superdex 75 10/30 column (Pharmacia Biotech Ab, Uppsala, Sweden) and eluted by 50 mM phosphate buffer ($NaH_2PO_4 \cdot H_2O$) pH 7.4, containing 150 mM NaCl.

Molecular Weight Estimation. SDS−PAGE was performed according to Laemmli (*13*). The protein bands were either stained with Coomassie brilliant blue or electroblotted onto a nitrocellulose mem-

* To whom correspondence should be addressed. Phone: 972-8-6596753. Fax: 972-8-6596742. E-mail: avigolan@bgumail.bgu.ac.il.
[†] Ben-Gurion University of the Negev.
[‡] The Hebrew University of Jerusalem.

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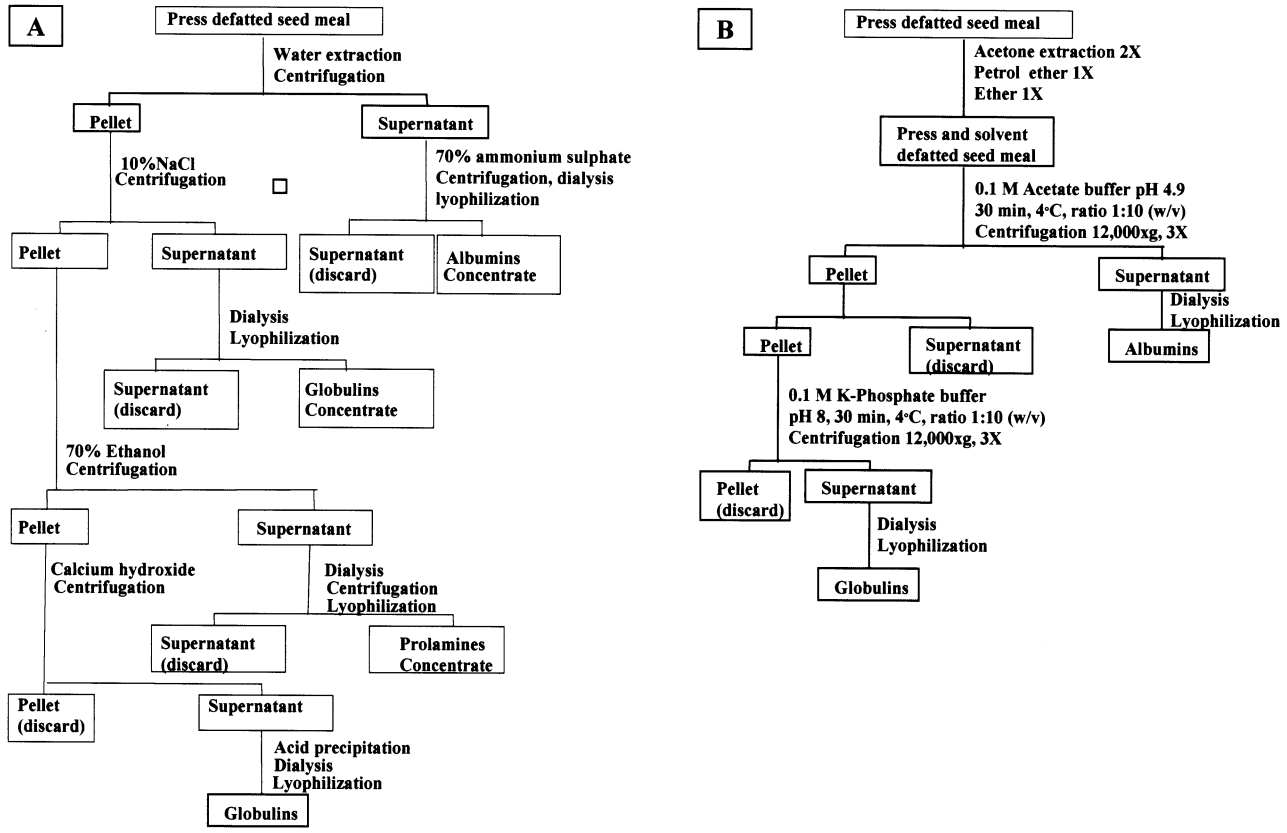

Figure 1. Flowcharts of fractionation protocols according to solubility of jojoba seed meal proteins: A, protein extraction according to Cardoso and Price (*10*); B, albumins and globulins extraction according to Crevieu et al. (*27*).

brane (Hybond-ECL, Amersham) (*14*). For immunochemical detection the membrane was incubated with a buffer containing a 1:2500 dilution of the primary rabbit polyclonal antibody raised against the Jojoba 25 kDa protein, as described by Livingston (*15*). After several washes, a secondary goat anti-rabbit immunoglobulin conjugated to peroxidase (Jackson Immunoresearch Laboratories, Inc.) was added at a 1:5000 dilution. The secondary goat antibody was detected by a peroxidase color reaction.

Protein Determination. Protein content in various fractions was determined by Bradford's method (*16*), using BSA as a standard, and by N-Kjeldhal method.

Amino Acid Analysis. Amino acid analysis of the proteins was done after hydrolysis in 6 N HCl at 110 °C for 20 h in vapor phase in a semiautomatic hydrolyzer from Knauer (Germany). Analysis was performed by precolumn derivatization of amino acids with FMOC (9-fluorenylmethoxycarbonyl chloride), which makes the amino acids fluorescent, and following separation of the derivatized amino acids by reversed-phase HPLC at 45 °C, in a gradient starting with 100 mM sodium acetate buffer pH 4.4/acetonitrile, in a starting ratio of 78:22 (v:v), respectively, to 100% acetonitrile within 1 h (*17*). The column used was a Superspher 60 RP8 LiChroCART (Merck).

Protein Microsequencing. The purified 25 kDa protein (Joj25) was microsequenced according to Matsudaira (*18*). The protein sample was submitted to SDS−PAGE. Following electroblotting of the gel onto polivinylidene difluoride (PVDF) membrane, the blot was stained with Coomassie brilliant blue, and the protein band was cut-out from the paper and sequenced directly on an Applied Biosystems 475A protein sequenator.

Spectrophotometric Assay of Protease and Protease Inhibitor. Enzymes and substrates were all obtained from Sigma Chemical Co. (St. Louis, MO). Proteolytic activity was measured using *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) and *N*-acetyl-L-tyrosine-*p*-nitroanilide (ATPNA) as substrates for trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1), respectively. In inhibition studies the following additional substrates were used: *N*-benzoyl-Ala-Ala-Ala-*p*-nitroanilide for subtilisin (EC 3.4.21.62) and elastase (EC 3.4.21.36), and *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide for thrombin (EC 3.4.21.5). In a standard assay, a reaction mixture contained 50 μL of the enzyme extract, the reaction buffer (0.1 M Tris−HCl buffer, pH 8.0, containing 0.01M $CaCl_2$) and 50 μL of 1 mM BAPNA in a final volume of 500 μL. The reaction was stopped by adding 150 μL of 30% acetic acid. Release of a *p*-nitroaniline group was measured spectrophotometrically at 410 nm.

Protease inhibitor was assayed by preincubation of the inhibitor, 20 μL containing various concentrations of the inhibitors in the range of 5 to 200 μg, with 50 μL of protease, in 380 μL of the reaction buffer at 37 °C for 15 min. The reaction was started by addition of the substrate and performed as described above. The remaining activity was expressed as percentage of the enzymatic activity in the absence of inhibitor.

Detection of Proteases on Polyacrylamide Gel. This was performed on PAGE according to Laemmli (*13*), except that gelatin (0.5 mg mL^{-1}) was copolymerized with the gel. Following electrophoresis under nondenaturing conditions, proteolytic bands were activated in the washed gel by incubation in Tris−HCl 100 mM, pH 8.0, containing 10 mM $CaCl_2$ at 37 °C for 30 min. The gel was stained with Coomassie brilliant blue R-250 and destained to reveal zones of substrate lysis. Clear zones on dark blue background indicate protease activity (*19*).

Detection of Protease Inhibitors on Polyacrylamide Gel. Protease inhibitors were identified on native polyacrylamide gel by visualization of the inhibitory activity according to Chavan and Hejgaard (*20*). After electrophoresis, the gel was incubated in 50 mM Tris buffer at pH 8.0, containing 100 μg/mL of either trypsin or chymotrypsin, at 37 °C for 10 min. The gel was rinsed 5 times in distilled water, then the substrate, *N*-acetyl-D,L-phenylalanine-β-naphthyl ester (APNE), and *o*-dianisidine were added, and the mixture was incubated for approximately 5 min at 37 °C. Color development was stopped by addition of 15% acetic acid. White bands indicated the position of the protease inhibitors on a purple background.

Protein Digestibility in Vitro. This was carried out according to Oshodi et al. (*21*). A sample of 31.25 mg of protein, either albumins or globulins, was dissolved in 5 mL of distilled water, and the pH was adjusted to 8.0 with 0.1 N HCl and/or 0.1 N NaOH, while stirring at

Table 1. Jojoba Seed Meal Protein Fractions According to Solubility

	extraction method[a]	
protein fraction	A (%)	B (%)
albumins	43.1	79.0
globulins	25.4	21.0
prolamines	9.5	none
glutelins	22.0	none

[a] Extraction method A (**Figure 1A**) was according to Cardoso and Price (*10*). Method B (**Figure 1B**) was according to Crevieu et al. (*27*). Protein content of the press and solvent defatted meals was 25% (N-Kjeldhal). Protein recovery was 83% and 56.9% by methods A and B, respectively.

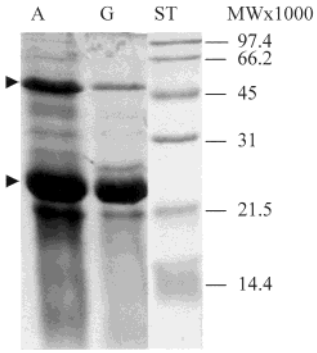

Figure 2. SDS–PAGE of jojoba albumins and globulins: lane A, albumins; lane G, globulins; lane ST, molecular weight markers. The 25 kDa protein (Joj25) and 50 kDa protein are indicated by arrows.

37 °C. A multienzyme solution, consisting of 1.6 mg of trypsin, 3.1 mg of chymotrypsin, and 1.3 mg of peptidase per mL, was maintained in an ice bath and adjusted to pH 8.0 as above. A 0.5 mL aliquot of the multienzyme solution was added to the protein sample solution with constant stirring at 37 °C. The pH of the solution was recorded 10 min after the addition of the enzymes solution. The in vitro digestibility was calculated using the equation of Hsu et al. (*22*)

$$Y = 210.46 - 18.1X$$

where Y is in vitro digestibility (%) and X is the pH of the sample suspension after 10 min digestion with the multi-enzyme solution. Casein and soybean protein isolate from Sigma were also analyzed as reference of known in vitro digestibility.

Statisitical Tests. Calculations of means, standard deviations, and Student's *t* test were carried out by SigmaStat for Windows, version 2.03, SPSS Inc.

RESULTS

Fractionation of Jojoba Proteins. The protein content of the Jojoba defatted seed meal was 25%. Because the most common approach to fractionate plant storage proteins is according to solubility, two methods of fractionation were compared (**Figure 1A**,**B**). A major part of the proteins were in the albumins and globulins fractions (**Table 1**): 43.1% and 25.4%, respectively, by method A (**Figure 1A**), and 79% and 21%, respectively, by method B (**Figure 1B**). Method B was used as the routine procedure to obtain these fractions. Separation of the albumins and globulins by SDS−PAGE (**Figure 2**) showed that there were two distinct protein bands of 50 kDa and 25 kDa in both. In addition, there were several other protein bands in each of these fractions (**Figure 2**). The 25kDa protein, named Joj25, being the major protein of the Jojoba seed meal, was further purified to homogeneity by ion-exchange chromatography on Q-Sepharose and HPLC separation on Superdex-75 gel filtration column, as described in Materials and Methods. The purified Joj25 was subjected to N-terminus sequence analysis and antibodies were raised against it and reacted specifically with Joj25 (**Figure 3**).

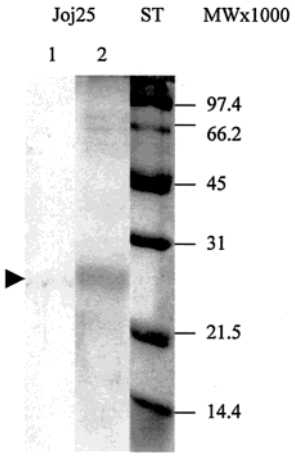

Figure 3. SDS–PAGE of the active band from PAGE trypsin inhibition gel reacted with antibodies against Joj25 on western blot, lane 1; and stained for protein by coommassie brilliant blue, lane 2; and lane ST, molecular weight markers.

Table 2. Amino Acid Composition of Jojoba Albumin, Globulin, and Joj25 Purified Protein[a]

	residues/mol[b]		
amino acid	albumin	globulin	Joj25
Asx[c]	31	29	25
Thr	17	15	9
Ser	16	16	5
Glx[c]	30	26	18
Pro	15	15	15
Gly	29	26	22
Ala	15	13	10
1/2-Cys	nd[d]	nd	nd
Val	17	16	16
Met	3	3	1
Ile	9	9	14
Leu	18	18	19
Tyr	9	8	5
Phe	10	10	8
His	5	4	4
Lys	10	9	6
Trp	nd	nd	nd
Arg	15	14	14
%EAA[e]	35.7	36.4	39.0
hydrophobicity[f]	848	878	1047

[a] Amino acid composition after 20 h hydrolysis in 6 N HCl at 110 °C. [b] The number of residues per mole of each amino acid is based on phenylalanine residues per mole. [c] Asx = Asp + Asn, and Glx = Glu + Gln. [d] nd, not determined. [e] %EAA, essential amino acids as mole percent of total amino acids, marked in bold letters. [f] According to Bigelow and Channon (*28*).

Amino Acid Composition of Jojoba Proteins. Amino acids composition of the albumins, globulins and Joj25 is shown in **Table 2**. The amino acid compositions of the albumins and globulins are similar but that of Joj25 shows a higher percentage of essential amino acids (39% versus approximately 36%). These values are comparable to raw soybean proteins (37.5%, according to USDA nutrition database) and higher than that in wheat flour whole grain (30.7%). The purified Joj25 was also more hydrophilic (1047) compared to the albumins (848) and globulins (878) (**Table 2**).

Joj25 from globulins was microsequenced and the 15 N-terminal amino acids were blasted against the SwissProt database. There were 18 significant hits. All of them related to

Table 3. Comparison of N-terminus Sequence of Joj25 and Major Database Blast Hit Proteins

Protein	Residue	Sequence	Residue	Identity	%positive
Joj25	1	SDTQNRPVLD INGNQ	15		
Invertase precursor	165	...ENNPVID VSSNQ	176	6/12	83
Beta lactamase	46	.DTQGE.YWD YNGNQ	59	9/14	64
Neuraminidase	299	....NRPVVD IN...	306	7/8	99
Sporamin B	40	......PVLD INGDE	48	7/9	99
Trypsin/Chymotrypsin Inhibitor	3	......PVLD VDGNE	11	6/9	99

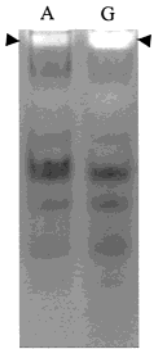

Figure 4. Gelatin–PAGE of jojoba albumins and globulins: lane A, albumins; lane G, globulins. Arrows indicate proteolytic activity bands.

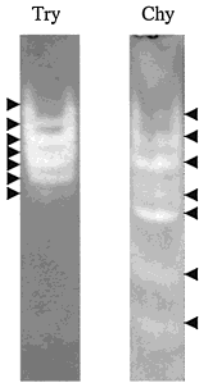

Figure 5. PAGE for protease inhibitors, trypsin inhibition (Try), and chymotrypsin inhibition (Chy) by jojoba albumins. Arrows indicate inhibitory activity bands.

5 proteins/enzymes (**Table 3**): β-lactamase, invertase precursor, neuraminidase, sporamin A and B, and trypsin/chymotrypsin inhibitor.

Proteases and Protease Inhibitors. Similar protease activity was noted in Jojoba albumins and globulins as shown on the gelatin PAGE (**Figure 4**). Both fractions contained also similar trypsin and chymotrypsin inhibitory activity (**Figure 5**). There were 7 inhibitor bands against chymotrypsin and trypsin in the albumins fraction. A similar pattern was obtained in the globulins with slight variations in intensity (not shown). The active bands from the trypsin gel were cut-out separately and ground to small pieces in an eppendorf tube containing minimal volume (just to cover the gel pieces) of 0.1 M Tris−HCl, pH 8.0. The buffered extracts were complemented with SDS and β-mercaptoethanol and run on an SDS−PAGE (**Figure 3** lane 2). The molecular weight of the protein was shown to be the same as that of Joj25. All trypsin inhibitory bands corresponded to 25 kDa protein band, indicating that the bands of activity on the nondenaturing gel (**Figure 5**) are isoinhibitors of the Jojoba trypsin inhibitor.

Table 4. Protease Inhibitory Activity of Jojoba Proteins[a]

	μg protein for 50% inhibition		
protease	albumins	globulins	Joj 25
chymotrypsin	65.1	80.2	20
trypsin	81.8	107.1	73

[a] All assays were conducted in the standard protease assay described in Materials and Methods, except for the following concentrations of the various enzymes (μg/assay): chymotrypsin, 10; trypsin, 2.5.

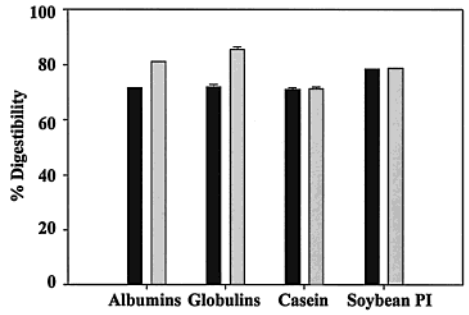

Figure 6. Digestibility in vitro of jojoba albumins and globulins in comparison to casein and soybean protein isolate (mean ± SD, $n = 3$). Black columns are of unboiled samples and gray columns are of boiled samples. Experimental error is indicated by standard deviation bars. The effect of boiling on the digestibility of jojoba proteins was statistically significant according to Student's t test ($p < 0.005$).

The protease inhibitory activity of Jojoba proteins is summarized in **Table 4**. Both albumins and globulins contained trypsin- and chymotrypsin-inhibitory activities. After purification, the Joj25 had stronger anti-chymotrypsin activity than anti-trypsin activity, 20 μg versus 73 μg, for 50% inhibition, respectively. There was no subtilisin and elastase inhibitory activity of Joj25, and thrombin inhibitory activity was unstable (data not shown).

Digestibility of Jojoba Albumins and Globulins. Evaluation of the nutritional quality of the Jojoba proteins was done by measuring the in vitro digestibility of the albumins and globulins (**Figure 6**). In the native albumins and globulins, it was 71.6% and 72.0%, respectively, when compared to casein (70.4%) and somewhat lower than soybean protein isolate (78.3%). Boiling of the Jojoba proteins for 10 min resulted in a significant increase in digestibility, up to 81.0% and 85.6%, respectively. The effect of boiling on casein and soybean protein isolate was insignificant (**Figure 6**).

DISCUSSION

Legume seeds and oilseeds are rich sources of economically important oil and proteins. The Jojoba seed is unique in that it is the only plant seed known to contain liquid wax. That is the reason for its wide introduction as an oil crop to arid zones. The defatted Jojoba meal is a protein-rich byproduct, which cannot be used as is for animal feed because it contains anti-nutritive factors, unless the proteins are isolated. However, the isolated proteins (albumins and globulins) contain proteases and protease inhibitors, which may be considered also as a source of bioactive compounds for medicinal purposes and possible food additives.

In this paper we have shown that the Jojoba seed meal proteins, when classified according to solubility (**Figure 1**), are composed mostly of albumins (water soluble) and globulins

(soluble in dilute saline) (**Table 1**). Both fractions contain proteolytic activity (**Figure 4**). Work is underway to characterize a protease from the albumins/globulins fractions specific for converting procaspase 3 to the active enzyme (unpublished data). It should be noted that multiple forms of endopeptidase activity were extracted from cotyledons of 27-day-old seedlings of Jojoba (*11*). The Jojoba seed meal contains also trypsin-, chymotrypsin-, and unstable thrombin-like inhibitors (**Figure 5** and **Table 4**). Work is underway to test their potential use in inhibition of hepatitis C virus NS3 serine protease (*23*).

Two major proteins, of 25 kDa and 50 kDa, were present in both the albumins and the globulins (**Figure 2**). The 25 kDa (Joj25) from the globulins was purified to homogeneity (**Figure 3**) and was shown to possess trypsin- and chymotrypsin-inhibitory activities (**Figure 5** and **Table 4**). The N-terminal sequence of this protein showed partial homology to database proteins (**Table 3**). Of special interest is the homology to a root storage protein from *Ipomea batatas* and the trypsin/chymotrypsin inhibitor from *Alocasia macrorrhiza*. The fact that Joj25 has a dual function, both as a major storage protein of the seed and as a protease inhibitor, suggests possible functioning in the dormant seed to inhibit proteolytic activity and may indicate some evolutionary efficiency. An analogous dual function of storage proteins was suggested also for the inflorescence bud storage protein of *Pistacia vera* L., which is also a dehydrin-like protein (*24*). The high hydrophilic nature of Joj25 (**Table 2**), indicates that this protein may have a function in water-holding and wetting during seed germination and imbibition.

The increased digestibility of the Jojoba albumins and globulins after boiling (**Figure 6**) may reflect inactivation of the protease inhibitors and denaturation of the proteins, making them more accessible to the proteases. Thus, the heat treatment has an obvious nutritional advantage. There could also be a beneficial use of a heat pretreatment of the seeds before oil extraction as recently published by Shabtai et al. (*25*) to inactivate protease inhibitors, as it was shown that addition of exogenous proteases to jojoba seeds facilitates oil extraction.

In conclusion, the Jojoba seed is a valuable source of proteins and enzymes: proteases, protease inhibitors, and other enzymes, e.g., acyl-CoA: fatty alcohol acyltransferase (*26*), involved in liquid wax biosynthesis. The presence of proteases and protease inhibitors goes beyond their function in the digestive tract. Jojoba offers also a source of proteases for industrial uses as tenderizers, as tissue breaking agents for extraction, and as proteolytic-activators of enzymes such as in apoptosis. Their inhibition of stored-product insect proteases and their cancer chemopreventive potential stimulate interest in plant-derived inhibitors, and the defatted Jojoba seed meal offers such a source.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; kDa, kilodalton; BAPNA, *N*-benzoyl-D,L-arginine-*p*-nitroanilide; ATPNA, *N*-acetyl-L-tyrosine-*p*-nitroanilide; APNE, *N*-acetyl-D,L-phenylalanine-β-naphthyl ester; PVDF, poly(vinylidene difluoride).

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