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Studies on karkade (*Hibiscus sabdariffa*): protease inhibitors, phytate, *in vitro* protein digestibility and gossypol content

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Karkade defatted flour extract had chymotrypsin inhibitor activity almost half that of trypsin (21.8 and 40.5 enzyme inhibitor activity units/mg protein, respectively). The protein isolate had a weak chymotrypsin inhibitor activity compared to that of tryprin (4.9–28.3 enzyme inhibitor activity units/mg protein, respectively). The trypsin inhibitor activity of the protein isolate was 70% of that of the defatted flour extracts. Extraction of trypsin inhibitor by citrate buffer (pH 4.6) gave a higher specific activity of trypsin than by phosphate buffer (pH 7.6), Tris buffer (pH 9.0) or distilled water. Heating the extract in boiling water for 10 min destroyed 66.1% of the trypsin inhibitor activity. Karkade protein isolate had lower free and total gossypol than did the whole seed and defatted flour. The *in vitro* protein digestibility of karkade defatted flour and protein isolate was lower than that of casein. Phytic acid was higher in karkade defatted flour than in soybean defatted flour. Copyright ©1996 Elsevier Science Ltd.

INTRODUCTION

The value of plant protein in supplying the protein needs in developing countries has been recognized in recent years. The seeds of *Hibiscus* are excellent sources of proteins (25.2%) and oil (21%) (Abu-Tarboush, 1996; Al-Wandawi *et al.*, 1984). However, *Hibiscus* seeds, like other seeds and legumes, contain antinutritional factors which may reduce their nutritional value or their protein digestibility. The importance of antinutritional factors is expected to increase in the years ahead along with increased use of proteins of plant origin (Esen, 1982). Protease inhibitors are among the most important antinutritional factors encountered in seeds. Trypsin inhibitors have been isolated in pure crystalline form from different plant sources (Kunitz, 1947; Roy & Rao, 1971). The presence of trypsin inhibitors in soybeans and other legumes was reported by Roy & Bhat (1974). Heat-inactivation of trypsin inhibitor in green soybeans was studied by Collins & Beaty (1980).

Gossypol is a constituent of cotton seeds (Murti & Achaya, 1975) and is toxic to monogastric animals (Berardi & Goldblatt, 1980). Schmidt & Wells (1990) reported the presence of gossypol in okra seeds and in seeds of three other plants in the Hibiscadeae tribe.

Mohamed *et al.* (1986) reported that soybeans and other oil seeds contain phytic acid which influences the bioavailability of trace minerals.

The purpose of this study was to assess the levels of trypsin and chymotrypsin inhibitors, levels of phytate, *in vitro* protein digestibility and gossypol content of karkade seed defatted flour and protein isolate. The effect of the type of buffers in the extraction of trypsin inhibitor and its thermal stability were also investigated.

MATERIALS AND METHODS

Preparation of defatted flour and protein isolate

Seeds of *H. sabdariffa* were obtained from the western region of Sudan. Seeds were cleaned by removing dust and plant debris. They were ground in a Waring blender to a flour consistency. Seeds were milled to pass through a 0.8 mm sieve using an ultra-centrifugal mill. The resultant flour was defatted with *n*-hexane at room temperature (22°C). The oil-free flour was desolventized in open air at room temperature. The defatted flour was further milled to pass through a 0.355 mm sieve.

The method of El-Tinay *et al.* (1988*a*) for protein extraction and the method of El-Tinay *et al.* (1988*b*) for protein coagulation at the isoelectric point were used for preparation of protein isolates.

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Preparation of the extracts

0.1 M sodium phosphate buffer (pH 7.6), 0.05 M sodium citrate buffer (pH 4.6), 0.1 M Tris (hydroxy methyl aminomethane) buffer (pH 7.0) and glass distilled water were used for extraction of defatted flour and protein isolate. One gramme of finely powdered defatted flour or protein isolate was blended with the appropriate buffers. All the above samples were shaken for 2 h at room temperature (Shaker Model 75, Burrell, USA) and then centrifuged at 4500 rpm for 20 min (Tlettich EBA35 Centrifuge, Germany). The supernatants were filtered to get clear solutions. All extracts were diluted 10 times (i.e. 1 ml to 9 ml) with distilled water or the appropriate buffer solutions. The aliquots containing 0.2, 0.4, 0.6, 0.8 and 1 ml were assayed for trypsin inhibitor activity (TIA) and for α-chymotrypsin inhibitor activity (CIA); 1 ml samples were used.

Trypsin inhibitor activity assay

Trypsin inhibitor activity was assayed following the method of Kakade *et al.* (1969, 1970) using 2% of *N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) as substrate. Trypsin type III from bovine pancreas (Sigma Chemical Co., St. Louis, MO, USA) was used for the assay. One trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance unit at 410 nm in 20 min for 10 ml of reaction mixture under the conditions described in this method, and the trypsin inhibitor activity as the number of trypsin units inhibited (TUI).

α-Chymotrypsin inhibitor activity assay

The method of Kakade *et al.* (1970) was employed for determining chymotrypsin inhibitor activity using bovine pancreas, Type II chymotrypsin (Sigma Chemical Co., St. Louis, MO, USA) and 1% casein (BDH Chemicals, Poole, UK) as substrate. One chymotrypsin unit (CU) was arbitrarily defined as an increase of 0.01 absorbance unit at 275 nm in 10 min for 10 ml of reaction mixture under the conditions described in this method, and the chymotryspin inhibitor activity as the number of chymotrypsin units inhibited (CUI).

Protein analysis

The protein content of the defatted flour was determined by the micro-Kjeldahl method (AOAC, 1990). Protein analysis for the extracted samples was carried out by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as standard.

Thermal stability of trypsin inhibitor activity

Citrate buffer extracts of defatted flour were heated to the boiling point for 10, 20, 30 and 50 min. Trypsin inhibitor activity was analysed according to the method of Kakade *et al.* (1969).

Phytic acid

The chromophore method for phytic acid determination proposed by Mohamed *et al.* (1986) was used.

***In vitro* protein digestibility (IVPD)**

The procedure of Hsu *et al.* (1977) as modified by Satterlee *et al.* (1979) was used. The drop of pH of casein (control) and the samples after 20 min hydrolysis by proteolytic enzymes was measured using an Orion Research Digital Ionanalyser/501 (USA). The enzymes used were trypsin type IX from porcine pancreas, chymotrypsin type II from bovine pancreas, peptidase type III from porcine intestine and protease type VI from *Streptomyces griseus*. All enzymes were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Gossypol content

Free gossypol was determined according to AOCS method Ba 7-58 (AOCS, 1974). Total gossypol was determined by the same method after acid hydrolysis using 0.1% oxalic acid. 0.6 M $CaCl_2$ was used to prepare the protein isolate from the seed defatted flour for gossypol determinations.

Determination of gossypol content

Acid hydrolysis of the bound gossypol
One gramme of the sample was put in a 100 ml volumetric flask. 25 ml of 0.1% oxalic acid was added. The flask was heated for 13 h at 75° C in a water bath. Then 25 ml of aqueous acetone (70%) and 5 ml of barium acetate (0.5%) were added and mixed and left to stand for 10 min. The mixture was filtered. The filtrate was then diluted to 100 ml with aqueous acetone.

Statistical analysis

Data were subjected to analysis of variance and mean separation was determined using Duncan's multiple range test as outlined by Steel & Torrie (1980).

RESULTS AND DISCUSSION

Trypsin and α-chymotrypsin inhibitor activities

Table 1 shows the variation of trypsin inhibitor activities in different buffers and in water. Extraction with citrate buffer (pH 4.6) gave the highest specific activity of TIA (40.5 units/mg protein) compared to water and the other buffers. The results may indicate variation in the extractability of the proteins having trypsin inhibitor activity. Our data agree with Roy & Bhat (1974)

Table 1. The effect of type of buffers in the extraction of trypsin inhibitor

Samples	Buffers	Trypsin inhibitor activity (unit/mg protein)
KSDF*	Phosphate (pH 7.6)	27.1 ± 0.35^{b}
KSDF	Citrate (pH 4.6)	40.5 ± 0.60^{a}
KSDF	Tris (pH 9.0)	13.4 ± 0.65^{c}
KSDF	Distilled water	27.1 ± 2.30^{b}

[a–c]Values are mean ± SE ($n = 3$). Values within a column that do not share a common superscript are significantly different at $P < 0.05$. *KSDF = karkade seed defatted flour.

Table 3. Trypsin and α-chymotrypsin inhibitor activities of karkade seeds defatted flour and protein isolate[a]

Samples* (1 ml)	Trypsin inhibitor activity (unit/mg protein)	α-Chymotrypsin inhibitor activity (unit/mg protein)
Karkade defatted flour	40.5 ± 0.60^{b}	21.8 ± 0.65^{b}
Soybean defatted flour	76.1 ± 1.10^{a}	57.2 ± 0.40^{a}
Karkade protein isolate	28.3 ± 0.30^{c}	4.90 ± 0.20^{c}

[a–c]Values are means ± SE ($n = 3$). Values within a column that do not share a common superscript are significantly different at $P < 0.05$.
*Samples extracted by citrate buffer.

who observed the same variation in TIA values when using water and phosphate buffer for the same soybean samples. Ikeda & Kusana (1978) reported that extraction of soybean with acetate buffer (pH 4.5) gave the highest specific activity of TI when compared to the other buffers used.

The effect of volume of extracts in the trypsin and α-chymotrypsin inhibitor activities of karkade defatted flour are shown in Table 2. Proteolytic inhibitor activities increased in both enzymes with the increase in the volume of the extracts. Karkade defatted flour extract had a higher TIA and α-chymotrypsin inhibitor activity (CIA) than protein isolate (Table 3). The TIA and CIA in protein isolate were 70 and 22.5% of that of the defatted flour, respectively. However, the TIA and CIA in karkade (defatted flour or protein isolate) were lower than in soybean defatted flour (Table 3).

Liener (1975) stated that many factors affect the TIA in legumes, such as heat treatment, germination, fermentation and legume protein isolates. Singh & Jambunathan (1981) reported considerable differences in the levels of trypsin and chymotrypsin inhibitors between two chickpea cultivars. The levels of TIA were higher in both seeds of chickpea cultivars than their CIA. The TIA in seeds of five dolichos bean cultivars were studied by Deka & Sarkar (1990). They found that all cultivars tested contained moderately high levels of TIA (values ranged from 2400 to 3200 TIU/g on a dry weight basis). Kortt (1983) studied the seed extracts of 27 varieties of *Psophocarpus tetragonolobus* from six regions of South-East Asia. All varieties showed trypsin and chymotrypsin inhibitor activities ranging from 22.2 to 42.5 and from 30.1 to 47.6 mg enzyme inhibited/g of seed meal for trypsin and chymotrypsin, respectively.

Protease inhibitors elicit diverse nutritional, biological and physiological responses in animals. The protease inhibitor in raw seed flours may cause pancreatic hypertrophy and reduce protein digestibility (Rackis, 1972).

Thermal stability of trypsin inhibitor

The effect of boiling water on inactivation of trypsin inhibitor is presented in Table 4. Unheated karkade defatted flour contained 41 TIU/mg protein. Trypsin inhibitor activity decreased during the first 10 min of heating such that 66.1% was inactivated. Further loss of inhibitor activity was very slight during the next 30 min, and at the end of 50 min 30% of the original TIA still remained. The inhibition of the enzyme activity in the sample and the loss of antiproteolytic activity on heating appear to show the heat labile nature of the trypsin inhibitor.

Most trypsin inhibitors in legumes and seeds can be destroyed or inactivated by proper heat treatment, although few (like buckwheat) are thermostable (Ikeda & Kusana, 1978). Much of the studies on trypsin inhibitor stability were carried out on soybeans. Collins & Beaty (1980) reported that TIA of fresh green soybean

Table 2. The effect of volume of extracts in trypsin and α-chymotrypsin inhibitor activities of karkade seed defatted flour[a]

Volume of extract[b] (ml)	Trypsin inhibitor activity		α-Chymotrypsin inhibitor activity	
	(unit/mg protein)	% inhibition	(unit/mg protein)	% inhibition
0.2(1.33 mg)[c]	19.4 ± 0.65	28.3	2.3 ± 0.20	5.0
0.4(2.67 mg)	29.3 ± 0.25	42.5	4.3 ± 0.30	9.1
0.6(4.0 mg)	31.5 ± 0.75	45.7	11.3 ± 0.45	23.7
0.8(5.33 mg)	36.2 ± 1.25	52.5	21.0 ± 0.25	43.3
1.0(6.67 mg)	40.5 ± 0.60	58.8	21.8 ± 0.65	45.6

[a]Values are means ± SE ($n = 3$).
[b]Samples extracted by citrate buffer.
[c]Weight of sample (mg) for the given volume.

Table 4. Trypsin inhibitor activity of karkade seeds defatted flour heated in boiling water[a]

Time samples held in boiling water (min)	Trypsin inhibitor activity (unit/mg protein)	Destruction of trypsin inhibitor activity (%)
Unheated	41.0 ± 0.20	—
10	13.9 ± 0.60	66.1
20	13.6 ± 0.50	66.8
30	13.0 ± 0.35	68.2
50	12.4 ± 0.30	69.8

[a]Values are means ± SE ($n = 3$).

was destroyed rapidly when the beans were heated in boiling water for up to 9 min. Tan & Wong (1982) stated that boiling the winged bean meal extracts for 5 min destroyed more than 75% of the TIA in the extracts. Koeppe *et al.* (1985) indicated that purified trypsin inhibitor of amaranth was very thermostable. It retained 20% of the original activity after 7 h at 100°C.

Phytic acid

Phytic acid contents in *H. sabdariffa* and soybean are shown in Table 5. *H. sabdariffa* had a high content of phytic acid compared to soybean, and the karkade protein isolate had a lower content of phytic acid than the seed defatted flour. The low content of phytic acid in karkade protein isolate may be a result of the differential solubilities of phytate and karkade protein during the preparation of protein isolate from the defatted flour by $CaCl_2$ extraction. Phytic acid is a food component implicated in reduced bioavailability of di- and trivalent minerals from cereals and legumes (Erdman, 1979). Ogun *et al.* (1989) reported the phytate content for four Nigerian cultivars of cowpeas. The mean values for the four cultivars were 1.2, 1.2, 1.1 and 1.1% for the raw, dehulled, cold water-soaked and hot water-soaked of the cowpeas, respectively. Deshpande *et al.* (1982) stated that the phytate content in the whole mature dry beans (*Phaseolus vulgaris* L.) ranged from 1.16 to 2.93%.

In vitro protein digestibility (IVPD)

Table 5 shows the *in vitro* protein digestibility (IVPD) of karkade and soybean. The mean values for protein digestibility of karkade defatted flour and protein isolate were 82.14 and 87.1%, respectively, and for soybean defatted flour 82.35%. The results indicate that casein was more easily digested with the proteases used than were karkade and soybean defatted flour. The *in vitro* protein digestibility for karkade defatted flour is similar to the digestibility (81.22%) of okra defatted flour (Bryant *et al.*, 1988).

Gossypol content

Free and total gossypol in karkade are shown in Table 6.

Table 5. Phytic acid and *in vitro* digestibility in karkade defatted flour and protein isolate

Products	Phytic acid (% dry wt basis)	Digestibility (%)
Casein	—	88.95 ± 0.1348[a]
Karkade defatted flour	2.37 ± 0.0491[a]	82.14 ± 0.0977[c]
Soybean defatted flour	1.69 ± 0.0346[b]	80.35 ± 0.2959[d]
Karkade protein isolate	0.17 ± 0.0088[c]	87.1 0 ± 0.1074[b]

[a–d]Values are means ± SE ($n = 3$). Values within a column that do not share a common supperscript are significantly different at $p < 0.05$.

Table 6. Free and total gossypol in karkade whole seed flour, defatted flour and protein isolate[a]

	Gossypol content (%)	
	Free	Total
Products	Mean ± SE	Mean ± SE
Whole seed flour	0.0680 ± 0.002	0.082 ± 0.003
Defatted flour	0.0550 ± 0.004	0.065 ± 0.002
Protein isolate	0.0036 ± 0.0004	0.004 ± 0.0004

[a]Values are means ± SE ($n = 3$).

The whole karkade seed flour had higher free and total gossypol content than did defatted flour and protein isolate. The lower gossypol content in protein isolate may be caused by the coagulation of the protein to its isoelectric point. Karkade protein coagulates at its isoelectric point (pH 4.5). The free gossypol content of the protein isolate at this pH is very low compared to the free gossypol content of protein extracts in other pH ranges. El-Tinay *et al.* (1988*b*) stated that a reduction of free gossypol in cotton seed flour was obtained by coagulating the protein to its isoelectric point. The presence of gossypol in okra seeds and in seeds of three other plants was reported by Schmidt & Wells (1990). They confirmed the existence of gossypol in plants outside of the Gossypieae tribe.

CONCLUSION

While the karkade seeds constitute an excellent source of protein, they also contain protease inhibitors, phytic acid and gossypol. However, this should not pose a problem in human nutrition if the seeds are properly processed. Further research is needed to investigate other antinutritional factors in karkade seeds.

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