Research Article

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Antioxidant activity in proteolytic enzyme digests of cashew (*Anacardium occidentale* L.) by-products

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Abstract

Membrane filtration of proteolytic enzyme digests and NaOH extracts of solvent extracted flours of cashew by-products resulted in reduction in antioxidant activities. Gel filtration of proteolytic enzyme digests of solvent extracted flours of cashew processing by-products on Sephadex G 25 revealed the presence of two peaks one immediately after the void volume and another later during elution. Reducing power, arginine and proteins content reduced in enzyme digests and alkali extracts after gel filtration and membrane filtration.

Keywords: Antioxidant activity, cashew, gel filtration, proteolytic digest

Introduction

Cashew, a crop introduced from Brazil is being grown in an area of 8.93 lakh ha in India, with a production of 6.95 lakh t. A total of 40 varieties have been released so far for cultivation in India. Most of these released varieties have been characterized for biochemical composition of kernel, apple, testa and shell (Nagaraja and Krishnan Nampoothiri 1986: Nagaraja 1987 a and b; 1992; 2000; 2006 and 2009). Antioxidant activities in cashew have been shown to be associated with number of small molecular weight compounds (Nagaraja, 2007). Numerous proteins and peptides of plant origin are known to exhibit cytotoxic effects (Hartmann *et al*., 2007). Functional properties of defatted cashew kernel flour (Nagaraja 2001; Alobo *et al*., 2009) and protein concentrate and isolate from cashew (Ogunwolu *et al*., 2009) have been studied. Cashew apple waste after fermentation has been recommended for blending with animal feed at 10 % level (Sundaram, 1986). Cashew nut meal at 15 % level in the diet did not affect the performance of broilers (Lopes *et al*., 2009). Cashew nut reject meal at 30 % blended with cassava peel meal at 10% was found to be appropriate for enhanced performance of growing pullets (Sogule *et al*., 2009). Oil from cashew kernel rejects has been extracted and refined to yield clear yellow oil whose properties compared well with the oil extracted from good kernels (Nagaraja 1998; Sini and Nair, 2009). Activated carbon prepared from cashew nut shells has been shown to absorb chromium from aqueous solutions (Tangiuank *et al*., 2009). Cashew apple juice has been shown to be a good source of reducing sugars which could be used as substrate for production of dextransucrase by *Leuconostic citreum* (Rabelo *et al*., 2009). Cashew apple juice is a good subrate for production of mannitol. (Fontes *et al*., 2009) and biosufactrant (Giro *et al*., 2009). It has been suggested that cashew apple powder could be useful in food industry as high dietary fiber ingredients (Costa *et al*., 2009). Cashew apple bagasse after alkali treatment could be fermented by *Saccharomyces cerevisiae* for the production of ethanol (Ponte Rocha *et al*., 2009). The molecular weight of the major globulin prepared from cashew kernel has been shown to be between 2,38,000 and 2,60,000 as estimated by different methods (Ventua and Filho, 1964). The IgE directed against 13S globulin and 2S albumin present in cashew kernel has been shown to be responsible for the food allergy exhibited (Teuber *et al*., 2002). IgE cross reactivity *In vitro* between cashew and walnut and cashewnut and peanut at the T Cell level has been demonstrated (Kulis *et al*., 2009).

As many of the cashew processing by-products have been analysed for various biochemical parameters,

and association of these biochemical components with antioxidant activity and desirable functional properties of defatted cashew kernel flour have been reported, an attempt has been made to study the antioxidant activities and associated biochemical components in proteolytic digests of flours of by-products of cashew processing and cashew apple powder after membrane / gel filtration which could help in developing functional foods.

Materials and Methods

Cashew kernel rejects, cashew kernel testa and cashew shell cake were obtained from M/s. Achal Industries, Mangalore. Cashew apples for the preparation of apple powder and apple pomace were collected from plantations maintained at Directorate of Cashew Research, Puttur. Solvent extracted flours were prepared as described earlier (Nagaraja, 2006). Cashew apple powder was prepared as described earlier (Nagaraja *et al.,* 2008).

Different flours (100 mg) was digested with Pepsin for 3 h followed by Trypsin (Sigma, 2690 units/ mg solid) and a-Chymotrypsin (Sigma, 15500 units/mg solid) for further 24 h and the enzyme digestion was terminated by boiling for 10 min on a boiling water bath. The enzyme digest was centrifuged and clear supernatant was made upto 5 ml with tripple distilled water and used for analysis. Suitable zero time blanks were also run as control with boiled enzyme (Akeson and Statmann, 1964).

An aliquot of the enzyme digest was filtered through membrane filters with a molecular cut off of 10,000, 50,000 and 2,00,000 using ultra filter units (USY 1, USY 5, and USY 20) procured from M/s. Merck. An aliquot (1.5 ml) of enzyme digests was filtered through membrane filters over a period of 24 h at 4 °C. Enzyme digests before and after membrane filtration were analyzed for absorbance at 280 nm, proteins (Oyama and Eagle 1956), arginine (Greinstein and Milton Winiz, 1961), reducing power (Yen and Chen 1995), nitric oxide scavenging and Fe chelating activity (Annie Shirwaikar *et al.,* 2006).

Different flour samples (1g) were extracted with 0.05 N NaOH (20 ml) for 1 h at room temperature and clear supernatant obtained after centrifugation was dialysed for 48 h at room temperature against distilled water and used for further analysis. The alkali extracted proteins were denatured by boiling on a boiling waterbath for 15 min and used for proteolysis and gel filtration studies.

Sephadex G 25 and G 100 were hydrated with excess distilled water for 30 min on a boiling water bath. The gel after swelling was cooled and packed into column of suitable dimension (h-56 cm, r-0.6 cm, Bed volume 63 ml) and equilbrated with distilled water. An aliquot (2 ml) of enzyme digests / alkali extract was loaded and eluted with distilled water (Flow rate 30 ml/h). Fractions (1.5 ml) were collected and monitored for absorbance at 280 nm.

Results and Discussion

Proteolytic enzyme digests of different flour samples were membrane filtered (10,000, molecular cut off) and the digest before and after membrane filtration was analyzed (Table 1). Membrane filtration resulted in considerable reduction in reducing power and absorbance at 280 nm. Reduction in protein was noticed only in case of defatted cashew kernel flour. Arginine content did not reduce due to membrane filtration. As peptides also react with protein reagent increased protein content in the filtrate is expected. The increase in some of the compounds in some flours after membrane filtration could also indicate the differential extent of proteolysis. In vitro digestibility of proteins of different released varieties has been shown to vary (Nagaraja, 1989).

Table 1. Analysis of enzyme digest before & after membrane filtration (10,000 Molecular weight cut off) n = 4

Flour	Protein,mg/100mg		Reducing Power A700/100mg		Arginine, mg/100mg		A280/100mg	
	Before	After	Before	After	Before	After	Before	After
A	19.92	16.50 (17.2)	1.847	1.551 (16)	2.332	2.527	25.81	25.74
B	12.89	20.93	5.14	3.09 (40)	2.202	2.569	39.61	30.85
C	8.96	4.68 (47.8)	33.93	24.71 (27.2)	0.175	ND	58.6	28.84
D	1.75	3.33	6.18	7.32	0.046	0.055	16.96	8.78
E	2.54	3.61	13.65	3.43 (75)	0.145	ND	18.53	11.79
F	4.50	5.06	52.93	40.44 (23.6)	ND	ND	57.51	58.67

Figures within parantheses indicate % reduction compared to before filtration,

ND - Not Detected.

A - Defatted flour of good cashew kernel, B - Defatted cashew kernel rejects ,C - MeOH extracted cashew kernel testa, D - Cashew apple powder from yellow fruits after autoclaving and drying, E - Cashew apple Pomace, F - Water extracted CNSL expelled cashew shell

The enzyme digests before and after membrane filtration was gel filtered on Sephadex G 25 and absorbance at 280 nm was monitored in the fractions (Figs. 1 and 2). Gel filtration profile of whole enzyme digest (Fig.1) revealed two fractions one immediately after void volume and another later. The fraction eluting immediately after void volume could be undigested protein while the fraction eluting later could be the enzyme digested products such as polypeptides. The gel filtration profile was similar in all the flours.

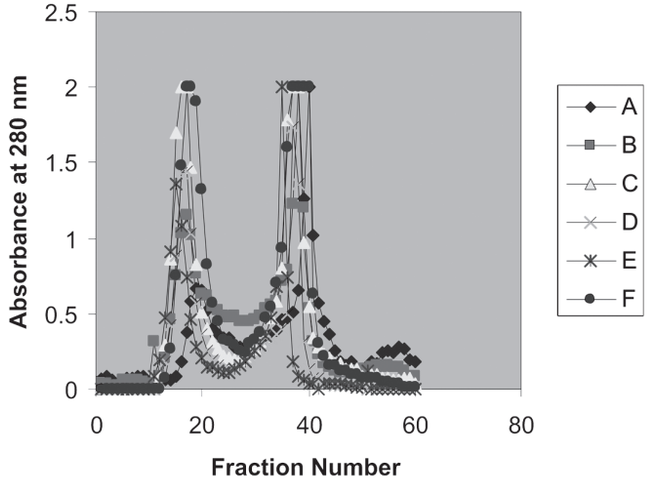

Fig. 1. Gel Filtration (G25) profile of whole enzyme digests of different flours

The gel filtration profile of enzyme digests of different flours after membrane filtration on Sephadex G 25 is presented in Fig. 2. In this case also, the gel filtration profile was similar. As the whole enzyme digests are membrane filtered to remove proteins with molecular weight more than 10000, the two fractions obtained during gel filtration could be peptides of different molecular size. The fraction eluting immediately after void volume could be peptide with molecular weight less than 10,000.

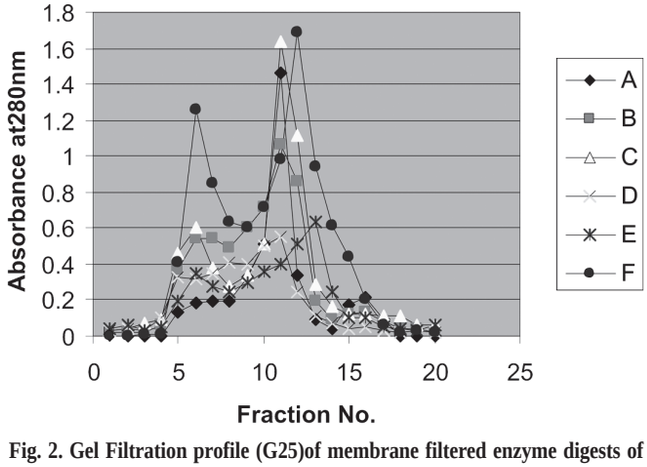

Fig. 2. Gel Filtration profile (G25)of membrane filtered enzyme digests of different flours

The pooled fractions after gel filtration (G.25) of membrane filtered enzyme digests of different flours were analysed (Table 2). The concentration of protein was higher in fraction 1 compared to fraction 2. Reducing power was higher in fraction 2 compared to fraction 1. Arginine content was less in fraction 1 compared to fraction 2. Protein, reducing power and arginine were not detected in pooled fraction 2 of membrane filtered enzyme digests of defatted flour of cashew kernel rejects and cashew apple powder of yellow fruits after gelfiltration on Sepheledax G. 25.

Table 2. Analysis of pooled fractions after gel filtration of membrane filtered (10,000 molecular weight cut off) enzyme digests of different flours

Flour	Protein, mg/ml		Reducing Power A700/ml		Arginine, ug/ml	
	Fra.1	Fra.2	Fra.1	Fra.2	Fra.1	Fra.2
A	0.707	0.097	0.075 (0.10)	0.78 (8.04)	81.96 (115.9)	27.32 (281.6)
B	0.750	ND	0.105 (0.14)	ND	97.57 (130.1)	ND
C	0.102	0.077	0.255 (2.5)	0.365 (4.74)	23.97 (235)	32.34 (420)
D	0.189	ND	0.165 (0.87)	ND	30.11 (159.3)	ND
E	0.097	0.111	0.155 (1.6)	0.18 (1.62)	30.67 (316.2)	22.86 (205.9)
F	0.092	0.068	0.355 (3.86)	0.385 (5.66)	12.82 (139.3)	13.94 (205)

Figures within parantheses indicate the values /mg proteins the values /mg proteins.
A to F Same as in Table 1, ND - Not Detected

As 0.05 N NaOH has been shown to extract proteins better from defatted cashew kernel flour (Nagaraja, 1987), defatted flours were extracted with alkali and membrane filtered. The alkali extracts before and after membrane filtration (10,000 molecular weight cut off) were analysed (Table 3). The absorbance at 280 nm, protein, reducing power and arginine contents in the extracts after membrane filtration were considerably reduced compared to alkali extracts before membrane filtration. The maximum reduction was noticed in defatted cashew kernel flour. Reduction in reducing power due to membrane filtrations indicated that the activity is associated with proteins as they are retained on the membrane filters due to the higher molecular weight. The molecular weight of cashew kernel proteins has been show to vary from 2,38,000 to 2,60,000 (Ventura and Filho, 1964).

Gel filtration profile of membrane filtered alkali extracts of different flours on Sephadex G.25 is presented in Fig.3. Two peaks one immediately after the void volume and another later during elution were observed. As the extracts were membrane filtered (10,000 molecular

Table 3. Analysis of 0.05 N NaOH extracts of flours before and after membrane filtration (10,000 molecular weight cut off)

Flour	Protein, mg/100mg		Reducing Power A700/100mg		Arginine, mg/100mg		A280/100mg	
	Before	After	Before	After	Before	After	Before	After
A	25.38	1.30 (94.9)	4.71	0.49 (89.6)	3.85	0.064 (98.3)	45.47	3.35 (92.6)
B	8.61	1.09 (87.3)	4.25	1.51 (64.5)	1.32	0.10 (92.4)	33.74	9.67 (71.3)
C	7.75	2.13 (72.5)	73.98	14.43 (80.5)	0.24	ND	70.21	30.26 (56.9)
D	3.74	1.97 (47.3)	17.85	9.86 (44.8)	0.097	ND	26.57	13.29 (50.0)
E	2.76	2.13 (22.8)	10.21	5.84 (42.8)	0.095	0.041 (56.8)	20.85	19.9 (4.5)
F	4.32	2.38 (44.9)	57.63	17.87 (69.0)	0.026	ND	64.61	44.49 (31.1)

Figures within parantheses indicate % reduction compared to before filtration.
A to F Same as in Table 1.
Values are mean of three individual estimations.

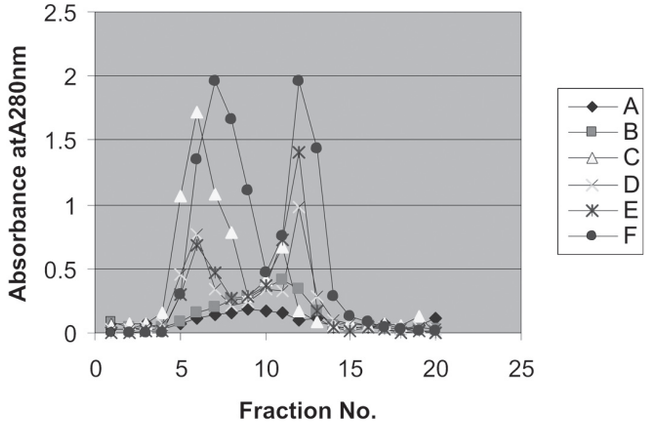

Fig. 3. Gel Filtration profile(G25) of membrane filtered 0.05 N NaOH extracts of different flours (10,000 mol wt cut off)

weight cut off), the molecular weight of the protein eluted could be less than 10,000. Pooled fractions after gel filtration of membrane filtered alkali extracts of flours were analysed (Table 4). In all the cases, the concentration of biochemical components analysed was higher in fraction 1 compared to fraction 2. In the case of defatted cashew kernel flour, three fractions were obtained.

The proteolytic enzyme digests of different flours before and after membrane filtration (50,000 and 2,00,000 molecular cut off) were analysed (Table 5). Filtration through membranes did not result in higher reduction in the concentration of biochemical components analysed as the molecular weight cut off of the filters was high. Similarly, the alkali extracts (0.05 N NaOH) of different flours were analysed before and after membrane filtration (50,000 and 2,00,000 molecular weight cut off) (Table 6). Per cent reduction in protein content, and arginine content

Table 4. Analysis of pooled gel filtered fractions of membrane filtered (10,000 Molecular Weight cut off) 0.05 N NaOH extracts of different flours

Flour	Protein,ug/ml			A_{280}/ml			Arginine, ug/ml			Reducing Power A700/ml		
	Fra.1	Fra. 2	Fra.3	Fra.1	Fra.2	Fra 3	Fra. 1	Fra. 2	Fra 3	Fra.1	Fra.2	Fra 3
A	80.29	8.92	7.14	0.282	0.101	0.053	13.12 (0.16)	4.67 (0.52)	3.79	0.168	0.144	0.17
B	64.23	-	-	0.539	-	-	12.83 (0.2)	-	-	0.228	-	-
C	73.15	41.03	-	1.061	0.430	-	4.67 (0.06)	3.79 (0.09)	-	0.37	0.288	-
D	83.85	105.26	-	0.475	0.437	-	2.92 (0.03)	ND	-	0.242	0.256	-
E	80.29	130.24	-	0.487	0.631	-	ND	2.04 (0.02)	-	0.244	0.27	-
F	67.80	78.50	-	1.156	1.151	-	ND	ND	-	0.584	0.482	-

Figures within parantheses indicate the values /ug protein .
A to F Same as in Table 1, ND - Not Detected

Table 5. Analysis of membrane filtered (50,000 and 200,000 molecular weight cut of) enzyme digest of different flours

Flour	Protein,mg/ml			Arginine,ug/ml			Reucing Power A700/ml		
	Before	1	2	Before	1	2	Before	1	2
A	6.33	6.03 (4.7)	6.31 (0.3)	445.8	464.0	514.7	1.11	0.47 (57.6)	0.44 (60.4)
B	6.56	6.26 (4.6)	6.17 (5.9)	510.4	527.3	485.1 (4.9)	2.97	2.03 (31.6)	1.93 (35.0)
C	3.45	1.92 (44.3)	2.44 (29.3)	44.3	ND	5.6 (87.3)	14.47	9.92	9.72
D	2.77	2.19 (20.9)	2.51 (9.4)	37.9	3.5 (90.8)	10.5 (72.3)	6.85	4.35 (36.5)	5.55 (18.9)
E	2.43	2.10 (13.6)	2.67	36.6	30.9 (15.6)	21.8 (40.4)	5.29	3.28 (38.0)	3.47 (34.4)
F	3.43	2.34 (31.8)	2.99 (12.8)	22.5	ND	ND	14.67	8.12 (44.6)	8.62 (41.2)

1 - Filtrate of 50,000 mol wt Cut off, 2 - Filtrate of 200,000 mol wt Cut off.
A to F Same as in Table.
Figures in paranthesis indicate % reduction compared to Before filteration
ND - Not Detected

Table 6. Analysis of membrane filtered (50,000 and 200,000 molecular weight cut off) 0.05 N NaOH extract of different flours

Flour	Protein,mg/ml			Arginine,ug/ml			Reucing Power A700/ml		
	Before	1	2	Before	1	2	Before	1	2
A	14.13	3.64 (74.2)	4.56 (67.7)	1207.3	204.8 (83.0)	254.5 (78.9)	1.18	ND	ND
B	5.59	2.73 (51.2)	3.30 (40.9)	408.1	152.1 (62.7)	186.3 (54.3)	0.52	0.14 (73.1)	0.26 (50.0)
C	4.87	2.07 (57.5)	3.64 (25.2)	68.2	ND	ND	33.36	13.7 (58.9)	21.7 (34.9)
D	2.03	1.25 (38.4)	4.87	36.97	ND	ND	6.58	5.4 (17.9)	4.8 (27.0)
E	1.78	1.64 (7.9)	2.00	5.69	ND	ND	4.22	3.3 (21.8)	3.4 (19.4)
F	2.27	1.86 (18.1)	2.48	ND	ND	ND	14.16	10.8 (23.7)	13.9 (1.8)

1 - Filtrate of 50,000 mol wt Cutoff, 2 - Filtrate of 200,000 mol wt Cutoff.
A to F Same as in Table 1.
Figures in paranthesis indicate % reduction compared to Before filteration
ND - Not Detected

did not vary between the two membrane filters with molecular cut off of 50,000 and 2,00,000. Per cent reduction in the reducing power of filtrate was, however, less when the extract was filtered through membrane with the molecular cut off of 2,00,000.

Gel filtration profiles of proteolytic enzyme digests of 0.05 N NaOH extracted native and denatured proteins of different flours on Sephadex G.100 are presented in Figs. 4 and 5, respectively. The proteolytic enzyme digested proteins eluted as two peaks one eluting immediately after void volume representing the undigested protein and another later representing the digested peptides. Gel filtration profile however, was similar with both native and denatured proteins.

Pooled fractions of gel filtrates of enzyme digests of alkali extracted proteins under native and denatured conditions were analysed (Table 7). Protein, arginine, reducing power nitric oxide scavenging activity, and Fe chelating activity were not detected in fraction 1 of defatted flours of good cashew kernels and cashew kernel rejects indicating the possibility of complete/better

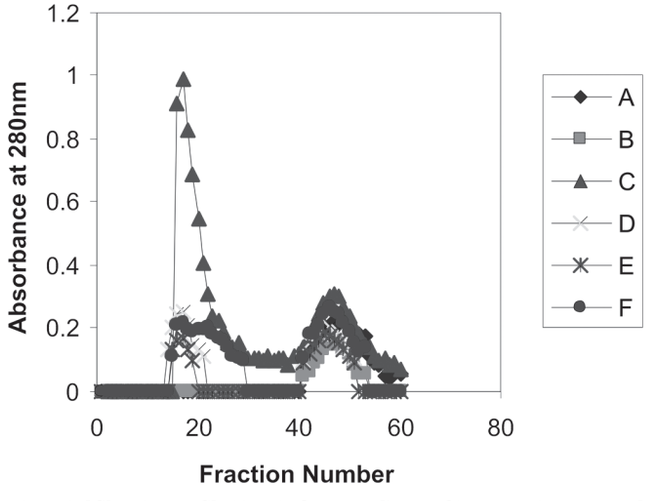

Fig. 4. Gel filtration profile (G100) of enzyme digests of 0.05 N NaOH extracted native proteins of different flours

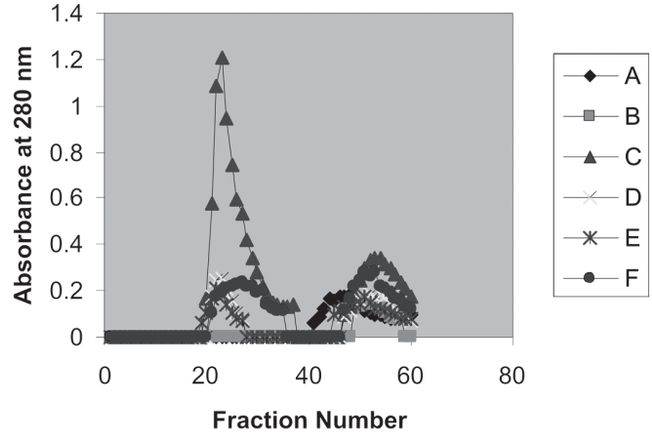

Fig. 5. Gelfiltration (G100) profile of enzyme digests of 0.05N NaOH extracted denatured proteins of different flours

digestion of proteins during proteolysis by proteolytic enzymes. The digestibility of protein either under native or denatured condition is poor in methanol extracted cashew kernel testa compared to other flours as evidenced by the higher content of proteins in the fraction 1 representing undigested proteins. Arginine content was higher in fraction 2 compared to fraction 1 of enzyme digests of alkali extracts of different flours. Nitric oxide scavenging activity and reducing power were detected in both the fractions except defatted flours of good cashew kernels and cashew kernel rejects. Iron chelating activity was not detected in fraction 1 of the enzyme digests of native proteins of all the flours. In the case of proteolysis of denatured alkali extracted proteins, Fe chelating activity was not detected in fraction 1 of defatted flour of good cashew kernels, cashew kernel rejects, methanol extracted cashew kernel testa and cashew apple powder of yellow fruits.

Gel filtration profile of denatured 0.05 N NaOH extracted proteins of different flours on Sephadex G.100 is presented in Fig.6. Denatured protein got separated and eluted as two peaks one immediately after void volume and another later during elution. This indicated the possibility of two protein fractions differing in their molecular weight. The profile remained similar in all the flours.

Table 7. Analysis of pooled Sephadex G 100 fractions of enzyme digests of native and denatured 0.05 N NaOH extracted proteins of different flours

Sample	Fraction	Protein, ug/ml		Arginine, ug/ml		Nitric Oxide Scavinging Activity, umols/ml		Reducing Power, A700/ml		Fe Chelating Activity, ug Fe chelated/ml	
		N	D	N	D	N	D	N	D	N	D
A	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	10.34	5.51	2.438	0.952	0.448	1.723	0.11	0.117	121.37	91.4
B	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	7.89	14.05	2.774	0.754	1.727	0.697	0.158	0.124	138.39	81.8
C	1	39.11	34.89	0.20	0.77	1.501	1.793	0.468	0.40	ND	ND
	2	8.81	13.87	2.686	0.652	1.773	2.152	0.266	0.147	106.0	80.72
D	1	10.02	12.02	0.578	0.482	1.331	1.195	0.285	0.212	ND	ND
	2	6.24	3.30	1.054	0.444	2.272	2.491	0.236	0.169	99.31	149.17
E	1	3.67	9.36	2.10	0.37	1.362	2.988	0.214	0.134	ND	40.69
	2	4.04	3.12	1.024	0.404	1.709	2.291	0.150	0.177	108.0	124.39
F	1	10.53	9.91	0.432	0.356	1.66	0.616	0.182	0.235	ND	50.8
	2	7.75	11.93	0.59	0.39	1.563	0.846	0.203	0.247	68.88	110.29
	CD(P=0.05)	2.39	1.75	0.662	NS	NS	0.51	0.03	0.09	22.9	32.75

A to F Same as in Table 1, ND - Not detected, NS - Not Significant, N - Native, D - Denatured

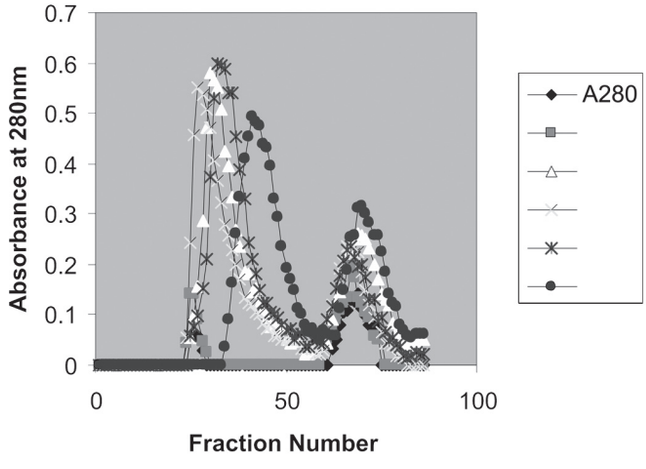

Fig. 6. Gel filtration (G100) profile of heat denatured 0.05 N NaOH extracted proteins of different flours

Gel filtration of alkali extract and proteolytic enzyme digests of solvent extracted flours of cashew processing by-products on Sephadex G.25 revealed the presence of two peaks one immediately after void volume and another later during elution. Reducing power decreased due to membrane filtration. DPPH scavenging activity was not detected either in the alkali extract or enzyme digests before and after gel filtration. The antioxidant activity in the protein digests is associated with both peptides and proteins.

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