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Food Chemistry 66 (1999) 241–247

Food Chemistry

www.elsevier.com/locate/foodchem

Studies of the physico-chemical properties and polyphenoloxidase activity in seeds from hybrid sunflower (*Helianthus annuus*) varieties grown in India

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Received 13 March 1998; received in revised form and accepted 22 December 1998

Abstract

Physico-chemical properties and polyphenol oxidase (PPO) activities of seven hybrid sunflower varieties commonly grown in Punjab state of India were studied. Seeds were studied for variations in physico-chemical properties, 100 kernel weight, density, bulk density, moisture, hull, oil, protein and ash content; expelled cake was studied for moisture, oil, crude fibre, ash and protein content and expelled-defatted cake for acid detergent fibre, lignin, moisture, ash and protein contents. PPO activity in all varieties, determined using pyrogallol as substrate at pH 6.5, varied between 0.212 and 0.294 Ab/min/mg protein. The PPO enzyme activity for MSFH-8 and Mega-363 was observed to be maximum at 55 and 60°C, respectively. The enzyme showed a K_m value between 1.01 and 1.968 mM for pyrogallol and V_{max} value between 0.21 and 2.0 Ab/min.

Keywords: Sunflower; Expelled-defatted meal; Physico-chemical properties; Polyphenol oxidase; Chlorogenic acid

1. Introduction

Sunflower (*Helianthus annuus*) has a great potential for meeting the ever-increasing demand for edible oil and protein by virtue of the high oil content and relatively high protein content of its meal. Sunflower is the third most important source of edible oil and fourth largest source of food and feed protein. Sunflower meal is primarily used as ruminant feed but its nutritional and functional properties make it potentially useful in human food. Polyphenol oxidase, present in sunflower, oxidises chlorogenic acid which constitutes more than 70% of the total phenolic compounds in sunflower seed (Carter, Gheyasuddin, & Mattil, 1972). The chlorogenic acid, in oxidised form, combines covalently with S and N groups of sunflower proteins at high pH or non-covalently through hydrogen bonding at low pH (Pierpoint, 1966, 1969; Sabir, Sosulski, & Kernan, 1974). The new complex thus formed between chlorogenic acid and protein imparts a dark green to brown colour to sunflower meal and protein isolates (Sabir et al., 1974). The colour depends on the extent of oxidation of chlorogenic acid which in turn depends on the amount of chlorogenic acid and PPO present in seeds. Many workers have attempted to obtain light-coloured protein isolates by either removing the chlorogenic acid with various solvents (Rahma & Narasinga Rao, 1981) or by inactivating PPO (Sayavedra & Montgomery, 1986). The present study reports the physico-chemical properties and PPO activity of sunflower hybrid seeds. Kinetic parameters such as K_m and V_{max} of different hybrid varieties of sunflower were also determined which can help in predicting the behaviour of the particular variety during protein extraction.

2. Materials and methods

2.1. Physico-chemical properties

Seeds of seven hybrid varieties of sunflower viz. PSFH-67, Jawala Mukhi U-5025, NSFH-592, SH-3322, Mega-363, GKFSH-2002, MSFH-8 grown in Punjab State were procured during the 1995 harvest. The produce of different varieties was cleaned and analysed for different physico-chemical properties. A known weight of samples (50 g) from each variety was dehulled manually percentage hull calculated. Ash, oil and protein contents of sunflower seed were determined using AOAC methods (Helrich, 1996). Bulk density (g/ml) of

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0308-8146/99/$ - see front matter
PII: S0308-8146(99)00057-6

sunflower seeds was measured as mass per unit volume. Density was determined by the kerosene displacement method. Sunflower seeds (500 g) were also expelled through a Single Screw Expeller (Komet, Germany) to obtain de-oiled meal. The expelled meals from each variety were analysed for moisture, oil, crude fibre, ash and protein contents. Expelled cake, after solvent extraction, was analysed for protein, ash, lignin and acid detergent fibre using the AOAC method (Helrich, 1990).

2.2. Chlorogenic acid and polyphenol oxidase enzyme

Chlorogenic acid from dehulled-defatted sunflower meal was determined using the method of Dorrell (1976). Polyphenol oxidase activity of various varieties was determined by the method of Raymond, Rakariyatham, & Azanza (1993) with slight modification. Michaelis constant (K_m) and maximum velocity (V_{max}) were determined with varied substrate pyrogallol concentrations (0.01–0.000625 M), as described earlier by Lineweaver and Burk (1934).

2.3. Preparation of enzyme extract

The sunflower seeds were dehulled manually, ground in a pestle and mortar and solvent extracted with petroleum ether (bp 40–60°C) in a Soxhlet apparatus for 8–10 h. The dehulled-deoiled meal (DDM) was again ground to pass through 60 mesh sieve. Sample (0.5 g) and glass powder (1 g) were added to 10 ml of buffer (pH 6.5) and again mixed thoroughly in a pestle and mortar. The solution was filtered through 4 layers of nylon cloth and filtrate was centrifuged for 15 min at 12,000 ***g***. To the supernatant, acetone (10 ml, −10°C) was added and the contents were kept in a refrigerator for 20 min for complete precipitation of the PPO proteins. The solution was again centrifuged for 15 min at 10,000 ***g*** and precipitates were collected and redissolved in 10 ml of buffer (pH 6.5). To the reconstituted solution, 2 ml of $CaCl_2$ (0.05 M) was added to precipitate out pectic substances. The solution was kept in a refrigerator for 15 min for complete precipitation of pectic substances and again centrifuged for 15 min at 10,000 ***g***. The supernatant was collected and stored in a refrigerator for further use as Enzyme Extract (EE).

2.4. Assay of enzyme activity

The standard reaction mixture consisted of 2 ml of 0.01 M pyrogallol; 1.5 ml of phosphate buffer prepared by mixing 68.5 ml of 0.2 M NaH_2PO_4 and 31.5 ml of 0.2 M $NaHPO_4$ (pH 6.5) and 0.5 ml of freshly prepared enzyme extract. The reference sample contained 2 ml of 0.01 M pyrogallol and 2 ml of phosphate buffer (pH 6.5). A Shimadzu UV-1601 spectrophotometer with TCC (electronic automatic temperature control) equilibrated at 37°C with enzyme kinetics software package was used to monitor change in absorbance at 334 nm for 10 min. One unit of activity was calculated from the slope of the curve which determined ΔA 334 nm/min due to oxidation of pyrogallol. The protein contents of EE were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and the specific activity was reported as Ab/min/mg of EE.

2.5. Enzyme kinetics

Michaelis constant (K_m) and maximum velocity (V_{max}) were determined with varied substrate pyrogallol concentration (0.01–0.02 M). Data were plotted as 1/activity vs 1/substrate concentration according to the method of Lineweaver and Burk (1934).

2.6. Effect of temperature on activity of PPO

To study the effect of temperature on PPO enzyme activity, the solution mixtures of pyrogallol (2.0 ml, 0.01 M) and buffer (pH 6.5) were kept in the cells of a spectrophotometer set at the required temperature with the help of TCC. The enzyme extract (0.5 ml) was added to the sample cuvette and immediately kinetics were recorded for 4 min at 334 nm to determine the activity at that temperature. Activities were measured at 0, 20, 30, 40, 50, 55, 60 and 70°C.

3. Results and discussion

3.1. Physico-chemical properties

The mean values for different physico-chemical characteristics of sunflower seeds from different varieties are reported in Table 1. Data revealed a wide variation in physico-chemical characteristics among different varieties. PSFH-67 had highest thousand kernel weight (65.58 g) and MSFH-8 had lowest (46.93). Bulk density and density for different varieties varied between 0.393–0465 and 0.694–0.8 g/cm^3, respectively. Hull contents of different varieties differ markedly and ranged from 22.3 to 28.0%. Wan, Barker, Clark, and Matlock (1979) observed bulk densities and hull contents between 0.31 to 0.42 g/cm^2 and 21–29%, respectively, for sunflower oilseed varieties grown in America. Oil content also showed significant differences among the varieties. U-5025 variety showed the highest oil content of 46.3% and GKFSH-2002, the lowest value of 37.92%. Protein, ash and moisture content of the kernels ranged from 22 to 24%, 2.69 to 3.47% and 4.22 to 5.68%, respectively. Robertson (1972) showed that the hull content of American sunflower varies from 22 to 28%. Kilara, Humbert, and Sosulski (1972) have reported the values for moisture, oil, protein, ash and crude fibre of sunflower kernel

to be 1.8, 55.4, 28.2, 3.6 and 2.4%, respectively. Kilara et al. (1972) have reported moisture, oil, protein, ash and crude fibre to be 6.5, 0.5, 55.5, 8.5 and 3.7%, respectively, whereas Lin, Humbert, and Sosulski (1974) reported these values as 7.5, 1.2, 60.1, 8.3 and 5.2, respectively, for other varieties of sunflower. The chemical compositions of the expelled sunflower meal obtained from different varieties are given in Table 2. Protein content of the sunflower meal from all the varieties varied from 28.6 to 31.0%. Sunflower meal obtained from the PSFH-67 variety showed the highest oil content of 16.23%, against a lowest value of 14% for NSFH-592. Crude fibre in sunflower meal from different varieties ranged from 15.28 to 16.93%, whereas moisture and ash content varied from 3.24 to 5.33% and 4.58 to 5.73%, respectively.

Acid detergent fibre and lignin of the defatted meal ranged from 27 to 32% and 9.2 to 13.56%, respectively (Table 3). The defatted meal from the U-5025 variety had the highest protein content (37%) and that from NSFH-592 the lowest (34.7%). Ash and moisture content varied from 5.0 to 6.7 and 2.1 to 2.97%, respectively.

3.2. Chlorogenic acid and polyphenol oxidase enzyme

It has been shown that the interaction of chlorogenic acid with protein is maximum at pH 3 and 7 and minimum at pH 5 (Saeed & Cheryan, 1989). So the determination of chlorogenic acid, which acts as substrate for PPO causing browning of protein isolates, is important. The optimum pH for the PPO enzyme is 5, hence the extraction conditions may be adjusted where there is

Table 1
Physico-chemical properties of seeds from various sunflower varieties[1]

Parameter	Varieties						
	PSFH-67	U-5025	NSFH-592	SH-3322	Mega-363	GKFSH-2002	MSFH-8
1000 Kernel weight (g)	65.58^{d}	60.23^{b}	60.42^{b}	62.21^{c}	49.0^{a}	56.56^{b}	46.93^{a}
Bulk density (g/cm^3)	0.436^{b}	0.45^{bc}	0.452^{bc}	0.454^{c}	0.465^{c}	0.443^{b}	0.393^{a}
Moisture (%)	4.22a	4.64^{ab}	4.96^{b}	4.47^{ab}	5.43^{c}	5.65^{c}	5.68^{c}
Hull (%)	25.63^{b}	24.0^{ab}	27.5^{cd}	22.3^{a}	26.16^{c}	27.0^{cd}	28.0^{d}
Ash (%)	3.18bc	3.28^{cd}	2.8^{a}	3.47^{d}	3.02^{b}	2.93^{ab}	2.70^{a}
Density (%)	0.704^{ab}	0.781^{cd}	0.746^{ab}	0.694^{a}	0.694^{a}	0.80^{d}	0.763^{bc}
Oil (%)	40.32^{ab}	46.3^{d}	43.54^{dc}	41.22^{ab}	40.22^{a}	37.92^{a}	42.0^{bc}
Protein (% N, 6.25)	23.9^{bc}	24.1^{c}	22.0^{a}	23.3^{ab}	23.0^{a}	23.2^{a}	22.3^{a}

[1] Values in rows with similar superscript do not differ significantly ($p < 0.05$).

Table 2
Physico-chemical properties of expelled meal (with hull) from various sunflower varieties[1]

Parameter	Varieties						
	PSFH-67	U-5025	NSFH-592	SH-3322	Mega-363	GKFSH-2002	MSFH-8
Moisture (%)	5.33^{d}	4.25^{ab}	4.72^{cd}	4.95^{cd}	3.24^{a}	4.47^{bc}	4.44^{bc}
Oil (%)	16.23^{c}	14.93^{ab}	14.0^{a}	16.18^{c}	14.23^{a}	14.97^{ab}	15.11^{b}
Crude fiber (%)	16^{bc}	15.76^{ab}	16.56^{cd}	15.28^{a}	16.1^{bc}	16.25^{c}	16.93^{d}
Ash (%)	4.87^{ab}	5.67^{b}	4.65^{a}	5.73^{b}	4.78^{a}	4.75^{a}	4.58^{a}
Protein (% N, 6.25)	30.3^{bc}	31.0^{c}	28.6^{a}	30.2^{bc}	29.9^{a}	30.3^{bc}	29.7^{a}

[1] Values in rows with similar superscript do not differ significantly ($p < 0.05$).

Table 3
Chemical properties of defatted meal (expelled whole seed including hull, extracted withpetroleum ether) of various sunflower varieties[1]

Parameter	Varieties						
	PSFH-67	U-5025	NSFH-592	SH-3322	Mega-363	GKFSH-2002	MSFH-8
Acid detergent fibre (%)	29^{b}	28.45^{ab}	32^{c}	27^{a}	29.45^{b}	31.28^{c}	32.05^{c}
Lignin (%)	10.84^{b}	10.48^{b}	12.4^{d}	9.2^{a}	11.8^{c}	12.03^{cd}	13.56^{c}
Moisture (%)	2.73^{bc}	2.29^{a}	2.09^{a}	2.64^{b}	2.67^{bc}	2.97^{bc}	2.75^{bc}
Ash (%)	5.89^{bc}	6.21^{cd}	5.12^{a}	6.69^{d}	5.39^{ab}	5.63^{ab}	5.01^{a}
Protein (% N, 6.25)	36.8^{bc}	37.0^{c}	34.7^{a}	35.5^{a}	35.0^{a}	36^{b}	35.0^{a}

[1] Values in rows with similar superscript do not differ significantly ($p < 0.05$).

least extraction of chlorogenic acid. Chlorogenic acid contents of dehulled-defatted meal obtained from different varieties ranged between 2.9 and 4.43% (Table 4). Variety SH-3322 showed highest chlorogenic acid, i.e. 4.434%. Dorrel (1976) reported that the chlorogenic acid in bred accessions and wild *H. annuus* from North America ranged between 1.42–4% and 1.58–2.7%, respectively. Rahma and Narasinga Rao (1981) reported a chlorogenic acid content of 3.26% in a Russian variety, EC-68414, grown in Southern India.

Table 4
Chlorogenic acid contents of different sunflower varieties of dehulled, defatted sunflower meal[1]

Variety	Chlorogenic acid (%)
PSFH-67	3.68^{cd}
U-5025	3.32^{b}
NSFH-592	3.35^{b}
SH-3322	4.43^{c}
Mega-363	2.91^{a}
GKFSH-2002	3.60b^{c}
MSFH-8	3.84^{dc}

[1] Values in rows with similar superscript do not differ significantly ($p < 0.05$).

Polyphenol oxidase activity in the seeds of different sunflower cultivars differs significantly. PPO activities varied from 0.212 to 0.294 Ab/min/mg protein of enzyme extract (average 0.253) using 0.01 M pyrogallol (Table 5). The activity expressed as PPO enzyme units, considering one unit of activity equivalent to 0.001 Ab/min/mg protein of EE, ranged from 212 to 294 units (average 252 units). Thus, the NSFH-592 variety showed the highest activity (294 units), followed by SH-3322 (289 unit) and the PSFH-67 variety showed the lowest activity (212 units). The K_m and V_{max} values were obtained by plotting 1/activity vs 1/substrate concentration as illustrated in Figs. 1–3. Inverse of the X intercept gives the K_m value and the inverse of the Y intercept gives V_{max}. The K_m value is a measure of the

Table 5
Activities, specific activities, K_m and V_{max} values of various sunflower varieties using pyrogallol as substrate

Varieties	Concentration (molar)	Abs/min	Activity (Abs/mg/min)	1/conc	1/activity	K_m (mM)	V_{max} (Ab)
GKFSH-2002	0.0100	0.0609	0.225	100	4.45	1.97	0.210
	0.0050	0.0419	0.155	200	6.47		
	0.0025	0.0290	0.107	400	9.35		
	0.00125	0.0199	0.0734	800	13.62		
	0.000625	0.0143	0.0527	1600	19.0		
U-5025	0.01	0.0556	0.288	100	3.48	1.55	0.255
	0.005	0.0421	0.218	200	4.59		
	0.0025	0.0257	0.133	400	7.53		
	0.00125	0.0196	0.101	800	9.87		
	0.000625	0.0150	0.0776	1600	12.9		
	0.000313	0.0102	0.0527	3200	19.0		
PSFH-67	0.01	0.0650	0.212	100	4.72	1.96	2.00
	0.005	0.0520	0.170	200	5.90		
	0.0025	0.0359	0.117	400	8.55		
	0.00125	0.0230	0.0749	800	13.3		
	0.000625	0.0170	0.0554	1600	18.1		
SH-3322	0.01	0.0560	0.290	100	3.45	1.50	0.285
	0.005	0.0451	0.233	200	4.29		
	0.0025	0.0313	0.162	400	6.18		
	0.00125	0.0227	0.117	800	8.52		
	0.000625	0.0168	0.0869	1600	11.5		
NSFH-592	0.01	0.0448	0.294	100	3.40	1.02	0.300
	0.005	0.0381	0.250	200	3.99		
	0.0025	0.0305	0.200	400	4.99		
	0.00125	0.0245	0.161	800	6.22		
	0.000625	0.0176	0.116	1600	8.65		
MSFH-8	0.01	0.0488	0.255	100	3.96	1.86	0.299
	0.008	0.0466	0.241	125	4.16		
	0.006	0.0529	–	166.6	–		
	0.004	0.0397	0.205	250	4.87		
	0.002	0.0299	0.155	500	6.47		
Mega-363	0.01	0.0225	0.0736	100	13.6	4.6	0.0942
	0.008	0.0184	0.0602	125	16.60		
	0.006	0.0162	0.0530	166.6	18.7		
	0.004	0.0118	0.0386	250	25.9		
	0.002	0.0079	0.0259	500	38.7		

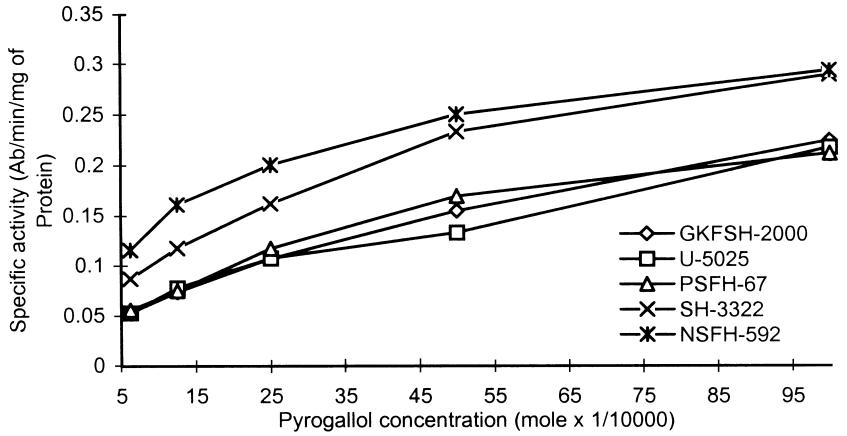

Fig. 1. Relationship between substrate concentration and specific activity.

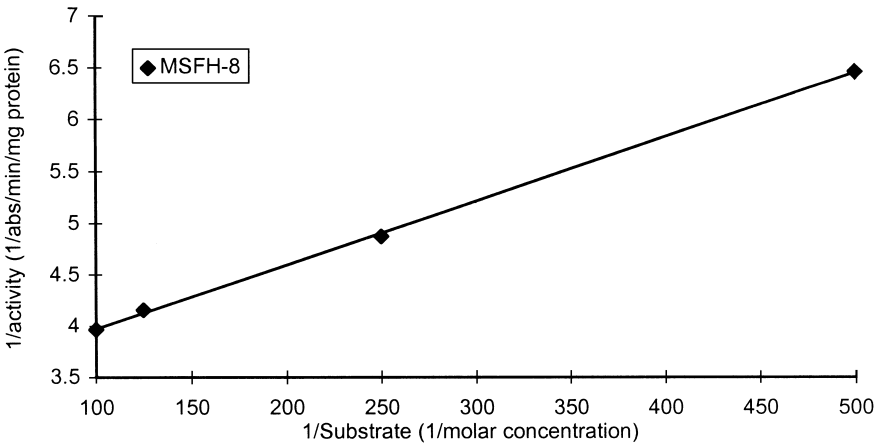

Fig. 2. K_m and V_{max} value of MSFH-8 using pyrogallol as substrate.

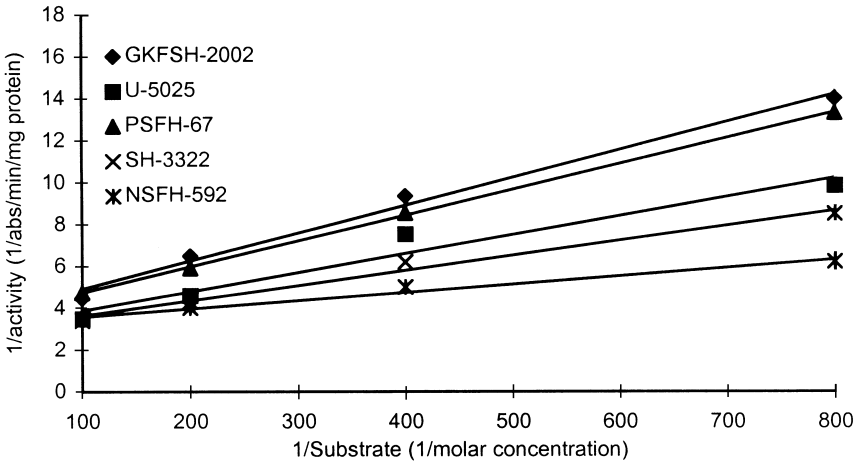

Fig. 3. K_m and V_{max} values of different sunflower varieties using pyrogallol as substrate.

affinity of enzyme for the substrate. A lower K_m value indicates a higher affinity of PPO for that substrate and vice versa. The different varieties of sunflower showed K_m values in the range of 1.015–1.968 mM of pyrogallol. A K_m value of 1.11 mM, determined by the Lineweaver and Burk (1934) method, has been reported for sunflower seed PPO using gallic acid as substrate (Raymond et al., 1993). The maximum reaction velocity (V_{max}) for different varieties of sunflower was observed to be in the range of 0.21 and 2.0 Ab/min. The PSFH-67

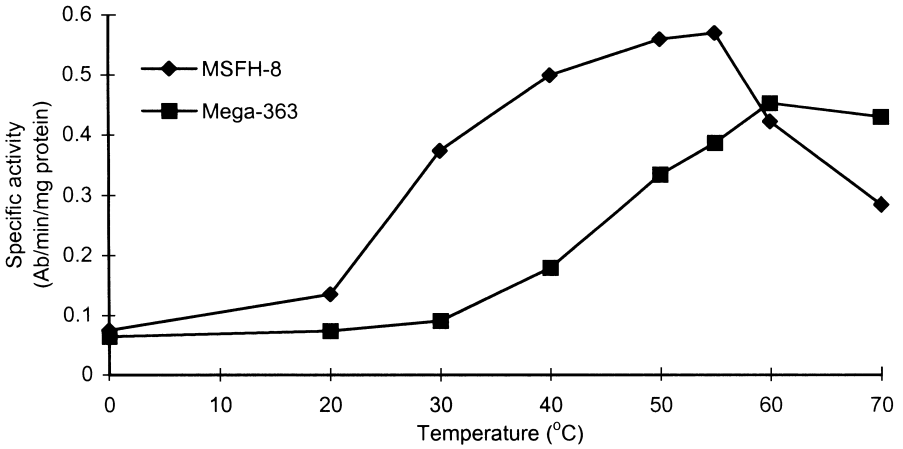

Fig. 4. Effect of temperature on the specific activity of PPO using pyrogallol substrate.

variety showed the highest V_{max} value of 2.0. Mega-363, with lowest chlorogenic acid, had lowest V_{max} and maximum K_m indicating lowest activity of enzyme and underlining its suitability for protein isolate processing. On the other hand, NSFH-592 and PSFH-67 with low K_m appeared to be problematic varieties; however, pH and temperature of extraction will be the main deciding factors as these will affect the activity of enzyme. During processing, the cells in sunflower seed are ruptured releasing polyphenol oxidase, which catalyses the oxidation of chlorogenic acid to *o*-quinone. The *o*-quinones are highly reactive and bind covalently with thiol or amino groups of proteins causing discolouration of protein (Pierpoint, 1969; Vaintraub & Kratch, 1989). The sunflower proteins are already deficient in lysine, this interaction further lowers the nutritive value because the new condensed products cannot be metabolized by humans (Earle, Van Etten, Clark, & Wolff, 1968; Synge, 1975).

The effect of temperature on PPO activity was studied for MSFH-8 and Mega-363 varieties only. PPO activity progressively increased with the increase in temperature up to 55°C in MSFH-8 and up to 60°C in Mega-363, respectively and afterward started decreasing (Table 6, Fig. 4). The enzyme showed maximum (100%) activities at 55 and 60°C for MSFH-8 and Mega-363, respectively, so the extraction of protein isolates with least browning may be done below or above these temperatures. The enzyme of MSFH-8 retained 13.11% of its activity at 0°C, 65.52% at 30°C and 98.2% at 50°C. At 70°C the enzyme lost 50% of its activity. The enzyme of Mega-363 retained 16.2% at 20°C, 20% at 30°C, 78.85% at 50°C and 85.4% at 55°C of its maximum activity. There is a sharp increase in the activity of enzyme in MSFH-8 from 0 to 30°C as compared to Mega-363 which can theoretically be processed for protein isolates at 20°C with least browning.

Table 6
Effect of temperature on the activity and specific activity of PPO of MSFH-8 using 0.01 M pyrogallol as substrate (rate time 0–4 min)

Temperature (°C)	MSFH-8		Mega-363	
	Activity (Ab/min)	Specific activity (Ab/min/mg)	Activity (Ab/min)	Specific activity (Ab/min/mg)
0	0.0272	0.0748	0.0340	0.0642
20	0.0490	0.135	0.0225	0.0736
30	0.136	0.374	0.0277	0.0907
40	0.182	0.500	0.0548	0.179
50	0.204	0.560	0.102	0.335
55	0.207	0.570	0.118	0.387
60	0.154	0.422	0.138	0.453
70	0.104	0.285	0.131	0.430

Acknowledgements

The investigation was supported by a grant from the Indian Council of Agriculture Research, New Delhi, to Dr. N. Singh.

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