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# Effect of varying pH on protein composition and yield of amaranth seed (*Amaranthus blitum*)

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**Abstract**

The isolation procedure of the seed proteins of *Amaranthus blitum* have been analyzed at different pH conditions. Qualitative studies were carried out by using electrophoretic technique sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Mainly four protein fractions *i.e.* albumin, globulin, prolamin and glutelin were obtained. Protein isolates were prepared by (a) extraction at different alkaline pH (9, 10, 11 and 12) and precipitation at pH 5 and (b) extraction at pH 9 and precipitation at different pH (4, 5, 6 and 7). The composition of isolates, prepared by method (a), depended on extraction pH. The isolate extracted at pH 8 was mainly composed of albumin and globulin, whereas at pH 9, 10 and 11 showed the presence of prolamin and glutelin. Electrophoretic pattern of different isolates had five major bands with molecular weight of 30, 45, 72, 84 and 90 kDt respectively. The increase of the extraction pH led to the increase in protein yield. With method (b) isolates obtained were variable in composition. At pH 7 albumin and prolamine were present, whereas at pH 4, 5 and 6 all 4 protein fractions were precipitated. According to the quantitative estimation of the albumin, globulin, prolamin and glutelin in the seed flour the contents were 26.4, 25, 5.81 and 42.7%, respectively. The results suggest that composition of protein isolates could be controlled by different extraction and precipitation pH.

**Key words**

*Amaranthus blitum*, Albumin, Globulin, Prolamin, Glutelin, Protein isolates

## Introduction

*Amaranthus* belonging to the family Amaranthaceae, comprises a series of wild, weedy and cultivated species and found worldwide in almost all agricultural environments. *Amaranthus* species have different centers of domestication and origin, being widely distributed in North America, Central America, and the South American Andes, where the greatest genetic diversity is found (Sun *et al.,* 1999; Xu and Sun, 2001). It is estimated that there are 87 species of *Amaranthus*: 17 in Europe, 14 in Australia, and 56 in America (Mujica and Jacobsen, 2003). Because of the paucity of studies on *Amaranthus* systematics, however, the number of species is still tentative. Some species are cosmopolitan, being both introduced and naturalized plants, with a weed-like behaviour, such as *A. retroflexus*, *A. hybridus*, *A. powellii*, and *A. viridis* (El Aydam and Bürki, 1997; Costea *et al.,* 2001b; Dehmer, 2003; Costea *et al.,* 2004). Amongst the cultivated species, *A. cruentus*, *A. hypochondriacus*, and *A. caudatus* stand out and are considered as pseudocereals, with a high seed protein content, a balanced amino acid composition, and a high lysine content (Barba de la Rosa *et al.,* 1992; Zheleznov *et al.,* 1997; Gorinstein *et al.,* 2001). These *Amaranthus* species are cultivated in different regions of South and Central America, India, and Nepal (Zheleznov *et al.,* 1997; Segura *et. al.*, 2002). The utilization of amaranth flour or their protein products is possible and could be useful, as food additives and ingredients (Tomoskozi *et al.,* 2008).

*Amaranthus* is often difficult to characterize taxonomically, as it has few useful distinguishing features amongst the large number of species. Moreover, hybridization is a common phenomenon in this genus, producing many interspecific hybrids that increase the taxonomic complexity (Lanta *et al.,* 2003; Wassom and Tranel, 2005). It is also difficult to delimit the distributional areas of the species, as many are cosmopolitan because of their capacity to adapt to the environment as well as the large quantity of seeds produced by the plant (Costea and DeMason, 2001; Costea *et al.,* 2004).

The nutritional quality of amarantha protein is very high because of its high content of lysine, arginine, tryptophan and sulphur containing amino acids. The lysine, a dominant content of amaranths, is twice in amount than that of wheat and three folds higher than that of maize. The amarantha protein belongs to the group of water soluble (albumin) and salt soluble (globulins) proteins (Segura Nieto *et al.,* 1992). On the other hand, protein content and amino acid composition depend on genotype and growing conditions. Juan *et al.* (2007) reported that seed proteins in *Amaranthus* are useful characters to discriminate between species and these characters show low environmental and evolutionary variability.

Electrophoretic techniques for seed protein profiles have already been used in the study of *Amaranthus* species (Gudu and Gupta 1988; Gorinstein *et al.,* 1991; Zheleznov *et al.,* 1997; Drzewiecki *et al.,* 2001). The properties of amarantha protein isolates and extracts were studied by several researchers (Cordero-de-los-Santos, 2005; Scilingo 2002) but very little data are available in the literature on composition of different protein isolates of this crop especially in the grain of *Amaranthus blitum.* According to the earlier reports, it has been suggested that properties of fractions in protein isolates and nutritional properties also depend upon preparation methods used and under different conditions.

The aim of this paper was to evaluate the protein extracts isolated by different solvents and analyses on the effect of extraction and precipitation (pH) on protein composition with the help of SDS-PAGE electrophoresis in *A. blitum*.

## Materials and Methods

**Protein extraction:** The seeds of *Amaranthus blitum*, were collected and grounded for flour. The defatted flour was separated by centrifugation and then air dried for 2 days at room temperature and finally stored at 4°C until used. Total protein was estimated according to the Lowry's method (1951).

Sequential extraction was performed according to the method of Sammour (1999) with minor modifications. *Amaranthus* flour (1 g) was extracted consecutively in four different solvents. First, the flour sample was extracted with distilled water (10 ml, w/v). The suspension was stirred at room temperature for 20 min. and then centrifuged at 8,000 rpm for 20 min. The supernatant was used as the extract 1(Albumin fraction). The remaining insoluble sample was mixed with aqueous 5% (w/v) NaCl (10 ml) solution, with the repetition of extraction procedure and the extract 2 was collected as globulin fraction. Subsequently extractions were followed with aqueous 70% (v/v) ethanol and aqueous 0.2% NaOH solution, the extract 3 as prolamin fraction and the extract 4 as glutelin fraction were obtained.

**Extraction at different pH and precipitation at pH 5:** The flour was suspended in water (10% w/v) and proteins were extracted at pH 8, 9,10 and 11 from the suspension and each pH were adjusted by adding 0.5 N NaOH. The suspensions were shaken for 30 min at room temperature and then centrifuged at 9,000 rpm for 20 min. The supernatants were adjusted to pH 5 with 1 N HCl and again centrifuged at 9,000 rpm for 20 min at 4°C. The precipitates were resuspended in water, neutralized with 0.1 N NaOH and freeze dried. Although four isolates obtained were termed as P8, P9, P10 and P11 according to the extraction pH.

**Extraction at pH 9 and precipitation at different pH:** The flour was suspended in water (10% w/v) and proteins were extracted at pH 9 according to the above described method. The supernatants of the extraction were adjusted to pH 4, 5, 6 and 7 with 1 N HCl. The precipitates were again resuspended in water, neutralized with 0.1 N NaOH and freeze dried. The freeze dried precipitates were termed as P4, P5, P6 and P7.

**Electrophoresis:** All gels were run in minislabs; (Bangalore Genei Vertical mini gel system). SDS-PAGE was carried out according to the method of Laemmli (1970). The runs were carried out in the following discontinuous buffer system: 0.5 M Tris-HCl pH 6.8 (4x stacking gel buffer), 1.5 M Tris-HCl at pH 8.8 (4x separating or resolving gel buffer) and 0.025 M Tris-HCl, 0.192 M glycine/1% (w/v) SDS, pH 8.3 for the running buffer. The protein samples (100 µl) were dissolved in the 100 µl of loading buffer( 0.5 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS/0.05 % (w/v) bromophenol blue and centrifuged at 5,000 rpm for 5 min: 25-30 µl lane$^{-1}$ supernatants were loaded in the gel. The electrophoretic runs were conducted for about 3-4 hr at a constant voltage of 80 V. The gels were stained with 0.5% R-250 Coomasie Brilliant Blue in water/methanol/acetic acid (4:5:1) for overnight and destained with water:methanol:acetic acid (9:9:1). The molecular weights of

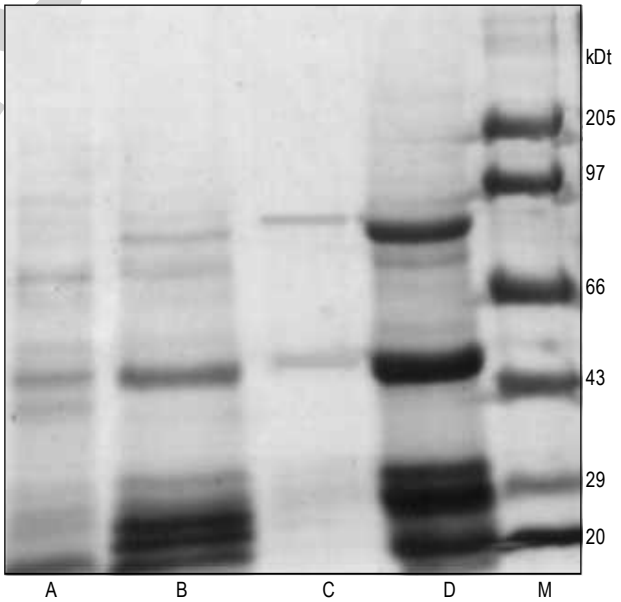


**Fig. 1:** SDS-PAGE of different fractions of amaranth protein: Lane A = Albumin, B = Globulin, C = Prolamin, D = Glutelin, M = Standard molecular weight proteins

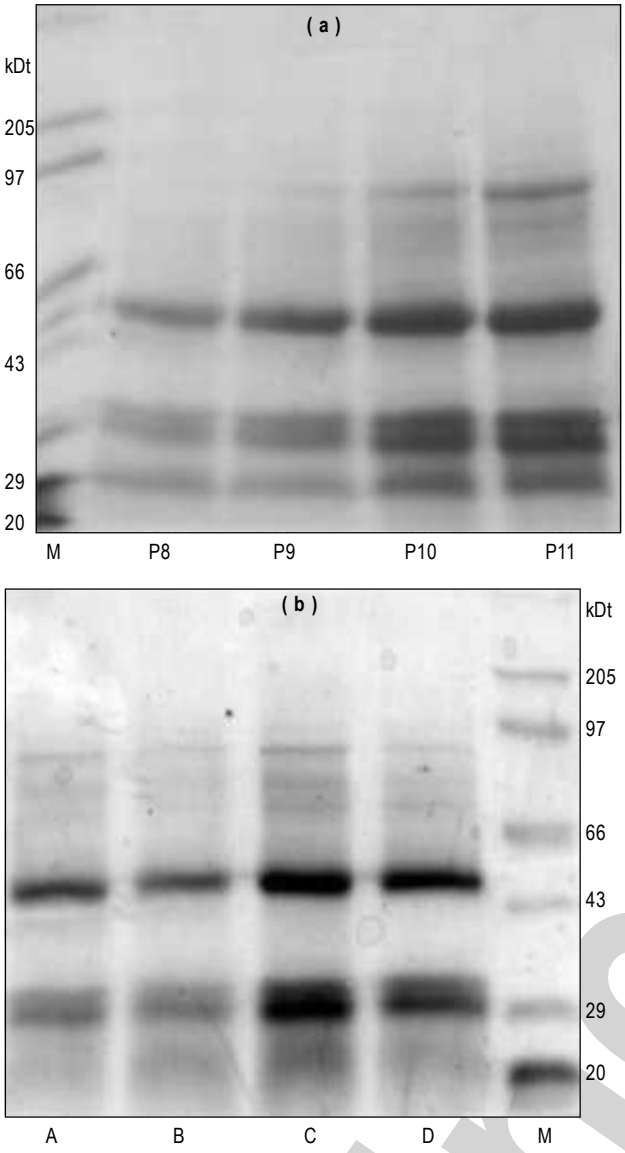


**Fig. 2:** SDS-PAGE of different protein isolates obtained (a) by extraction at different pH and precipitation at pH 5. (b) by extraction at pH 9 and precipitation at different pH. M, standard molecular weight proteins

polypeptides were calculated by using the protein standard molecular marker.

## Results and Discussion

According to the results, the amount of total protein was 12.17% of seed flour and the content of albumin, globulin, prolamin and glutelin were 3.22, 3.05, 0.70 and 5.20 g 100 $g^{-1}$ seed flour respectively where the content of glutelin was maximum in comparison to all the protein fractions and it was approximately 7 folds higher than prolamin, which was lowest in total protein. In SDS-PAGE, the amaranth seed flour protein extracted with distilled water (albumin fraction) showed the dominant bands of low molecular weights of ≤32 kDt (Fig. 1, lane-A) and some components of intermediate

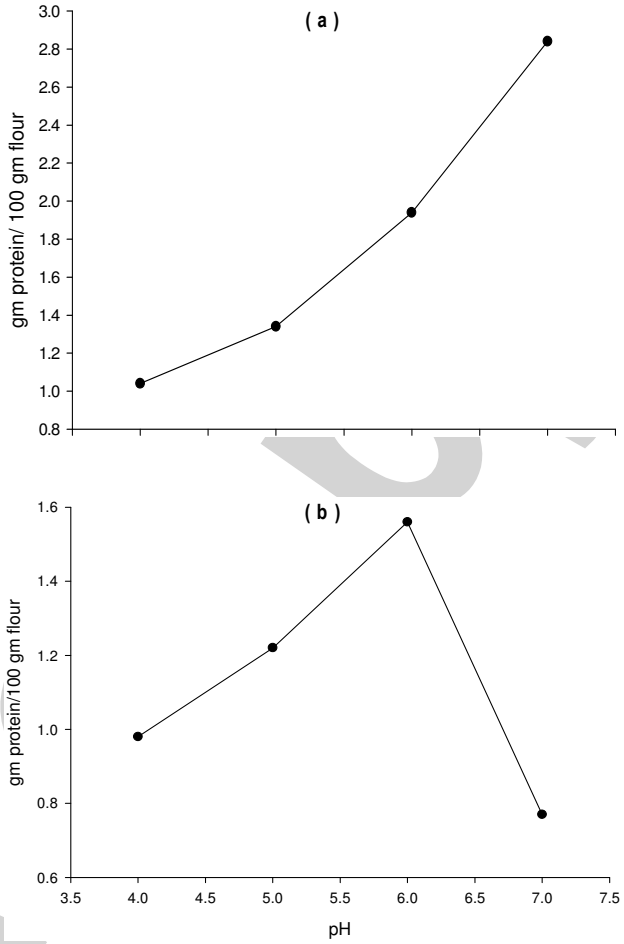


**Fig. 3:** Yield of protein isolates obtained (a) by extraction at different pH 5 (b) by extraction at pH 9 and precipitation at different pH

molecular mass between 43-70 kDt, which is parallel to the findings of Segura-Nieto *et al.* (1992). In globulins (Fig. 1, lane-B) the electrophoretic profile showed the band distribution similar to that already reported by Segura-Nieto *et al*. (1994) in *A. hypochondricus*. Electrophoretic separation of this lane included some intermediate components of low molecular mass of below 29±1 and 54±1 kDt and of a high molecular mass 70 and 80 kDt; this indicated the presence of different subunits of globulin. In prolamins (Fig. 1, lane-C) the most dominant band was about 54±1 kDt and the precipitation of a secondary band of higher molecular mass of about 85 kDt. The distribution of polypeptides of glutelin (Fig. 1, lane-D) showed similar bands to that of combination of globulin and prolamin. One prominent band of 55±1 kDt and of intermediate polypeptide mass of 70 and 80 kDt along with an abundant protein band of about 85 kDt were observed. It is assumed that the fractions were extracted at non extreme conditions; therefore possibly, it could have been considerably contaminated with globulins and albumins.

**Proteins extracted at pH 8, 9, 10 and 11 and precipitated at pH 5:** These isolates referred to as P8, P9, P10 and P11 according

to the extraction pH and together they had a protein concentration in the range of 1.02-2.84 g 100 $g^{-1}$ of flour (Fig. 3a). The yield of protein increased corresponding to the extraction pH (P8-11). The isolates were analyzed by SDS-PAGE, and the results are shown in Fig. 2a. By comparing the profiles of the isolates with those of the partially purified fractions, presence of low molecular mass peptides (32, 29 and 20 kDt) typical albumins were in higher proportion and band of minor polypeptides mass 54±1 kDt predominates (Fig. 2a, lane P8). The result showed that the protein extracted at pH 8 contains high proportion of albumins. The electrophoretic pattern of the other isolates P9, P10 and P11 (Fig. 2a, lane P9, P 10 and P 11 respectively) indicated that all isolates commonly share the major bands of 20 ±1, 29±1, 32±1, 54 and 85 kDt respectively. One minor band of high molecular mass of 80 kDt was also observed in P11. The presence of high molecular mass (85 kDt) evidenced the presence of globulin, prolamin and glutelin mainly at pH > 8.

**Protein extracted at pH 9 and precipitated at pH 4, 5, 6 and 7:** Protein isolates obtained at pH 4, 5, 6 and 7 were referred as P4, P5, P6 and P7. The effects of precipitation pH are shown in Fig. 2b. Proteins precipitated at different pH had protein concentration in the range of 0.98-1.56 g 100 $g^{-1}$ of flour and the protein yield was significantly higher at lower pH (4-6) than greater pH value (pH 7) (Fig. 3b). The electrophoretic profile of the isolates showed that the isolates P4, P5 and P6 shared all bands of the four fractions and showed the expression of polypeptides 29-32, 54±1 and 83±1 kDt (Fig. 2b, lane P4, P5 and P6). In lane P7 peptides of low molecular masses 29-32 and 55±1 kDt were present in addition to a minor band of about 83 ±1 kDt in lane P6. This presence indicated the partial presence of globulin. Lane P4, P5 and P6 (Fig. 2b) showed the presence of all the four fractions of protein as indicated the predominance of low molecular mass bands. Absence of band of high molecular weight in lane D revealed the absence of globulin and glutelin and predominance of albumin and prolamin. The result concludes the presence of albumin at all pH and globulin, prolamin and glutelin at pH 4, 5 and 6.

There are several procedures of protein identification, majority of them are very time consuming but have been successfully applied for the identification of proteins in numerous biological systems (Cordwell *et al.,* 2001; Mann *et al.,* 2001). However, the advantage of present proteomic method is mainly use of smaller amount of protein samples and speedy protein identification. Based on the above method, some major proteins were characterized with SDS-PAGE electrophoresis. In the present finding, major proteins such as albumin, globulin, prolamin and glutelin were identified. Amount of protein extracted at pH 8-11 varied according to the extraction pH and showed the amount in the range 1.04-2.84 gm of protein 100 $g^{-1}$ seed flour. The contents of albumin, globulins, prolamins and glutelins were 26.4, 25, 5.81 and 42.7% of the total seed protein respectively and showed the variation from the results obtained by Segura-Nieto *et al.* (1992). *Amaranthus* seed proteins are composed of albumins, globulins and glutelins in similar proportions and prolamin in minor amount (Abugoch *et al.,* 2003). This can be assumed that variation in protein content could be due to methodological or plant varietals differences or differences in ecological conditions (Sammour, 1999). Based on solubility of grain proteins in different solvents, the dominating protein fractions (albumin, globulin and glutelin) were accounted for 94.1% of the total seed protein whereas prolamin amount was very low in comparison to other fractions. However, prolamin content was 5.81% that is almost two fold higher than the value (2.2%) obtained by Segura-Nieto *et al.* (1992). In our findings the extraction conditions was very strictly maintained as suggested earlier (Kim and Bushuk, 1995) and the isolates and protein fractions were analyzed by SDS-PAGE. In the pattern of albumin (Fig. 1, lane-A), peptides of low molecular mass (<32±1 kDt) was predominated with the presence of components of intermediate molecular mass (54±1 kDt) which is agreement with previous studies (Konoshi *et al.,* 1991; Segura Nieto *et al.,* 1994; Marcone and Yada, 1992). Amaranth proteins expected to be highly heterogenous and heterogenesity could result from several causes and the results in fragmentation of subunits are expressed on the basis of acidic and basic polypeptide chain (Gorinstein *et al.,* 1991; Barba de la Rosa *et al.,* 1992b). In globulins (Fig. 1, lane-B), the electrophoretic profile showed bands of different mobility and showed the agreement with earlier findings of Segura- Nieto *et al.* (1994). The distribution of high molecular mass and polypeptides of intermediate molecular mass of 29±1 and 54±1 kDt draw the possibility of a typical behavior like disulphide bonded acidic and basic chains in 11S storage protein as found in *Pisum, Vicia* and *Lenses* (Barba de la Rosa *et al.,* 1992). Gorinstein *et al.* (1991) have reported that amaranth globulins are comprised of only one major band of 16±2 kDt, whereas in our findings, electrophoretic profile showed the distribution of bands $\leq$32 kDt. Prolamin expressed the distribution of bands about 54±1 kDt with the precipitation of a high polypeptide mass of 85 kDt. The electrophoretic separation of prolamin fraction in amaranth also showed the similarities with distribution pattern of barley, maize and wheat, consisting of bands ranging from 50-90 kDt. According to the separation of polypeptides masses of glutelin fraction was similar to combination of globulins and prolamins in terms of molecular weight. Initially prolamines and glutelins were thought to be distinct group of proteins but recently many glutelin proteins are shown to be closely related with prolamins, but considerable difference is solubilities and presence of highly heterogenous major bands. Electrophoretic analysis of protein isolates extracted at different pH is mostly based on the molecular weight of about 54±1 kDt. By comparing the profiles of isolates with those of partially purified fractions it was sorted out that low molecular mass peptides, typical albumin are present in higher proportion in all the isolates whereas expression of polypeptide of high molecular mass of about 80±1 and 85±1 kDt in isolates P9, P10 and P11 indicate that globulins, glutelins and prolamins are extracted mainly at pH higher than 8. The electrophoretic profiles of precipitation at different pH revealed all four fractions in lane P4 and lane P5 (Fig. 2b). The protein yield was significantly higher at lower pH 6 than at

pH 7. On the other hand the electrophoretic profiles of the isolates showed disappearance of high molecular weight bands at increasing pH 6-7. It has been assumed that the increase of the extraction pH can be induced the conformational changes in the proteins. In our findings composition of amaranth protein isolates could be modified by changing the precipitation pH as the observation of selective precipitation of globulin peptides and some lower molecular mass polypeptides at pH 6 and 7, where glutelin polypeptides are hardly detected. The results obtained here ensure that composition and structural changes of amarantha proteins depend on extraction pH as it is suggested that albumin and globulin are mostly extracted at pH 8, whereas at pH>8, albumin, globulin, glutelin and prolamins are obtained. According to the results, the composition of protein isolates depends on extraction and precipitation pH. It is confirmed that albumin and globulin are dominant subunits of amarantha protein.

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