

## Isolation of Wheat Puroindoline-b from Flour by Preparative Acid Electrophoresis

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Grain hardness is the most important characteristic affecting the quality of common wheat end-products. In fact, it influences milling and properties of flours; soft wheat flour is typically used for biscuits (cookies) and cakes and hard wheat flour is typically used for bread. Wheat grain hardness appears to be determined by the degree of adhesion between the starch granules and the protein matrix, which is regulated by a protein called friabilin, isolated from prime starch granules (Greenwell and Schofield 1986). Friabilin is present in larger amounts in soft than in hard wheats and consists of two proteins (Jolly et al 1993; Morris et al 1994), the N-terminal sequences of which were identical to those of two binding lipids proteins called puroindoline-a (Pin-a) and puroindoline-b (Pin-b) (Gautier et al 1994). Hardness is the result of the expression of one major gene, *Ha*, located on the short arm of chromosome 5D, controlling the occurrence of friabilin in the whole grain (Jolly et al 1993). Further evidence supporting the relationship between friabilin and *Ha* locus comes from the observation of several mutations in Pin-a and -b that are consistently associated with hard endosperm texture (Giroux and Morris 1997, 1998).

Pins are cysteine-rich proteins (10 cysteine residues forming five disulfide bridges) with a unique tryptophan-rich domain and a molecular mass of  $\approx 13,000$  (Douliez et al 2000). Furthermore, they are strongly basic proteins with calculated isoelectric points of pI 10.5 for Pin-a and pI 10.7 for Pin-b (Gautier et al 1994).

Methods for the isolation of Pins from flour (Blochet et al 1993) and starch granules (Jolly et al 1993; Morris et al 1994; Oda and Schofield 1997) were previously reported in the literature. In these methods, purification of Pins was achieved combining Triton X-114 partitioning, gel filtration, and cation-exchange chromatography techniques.

Here we present an improvement of the Blochet et al (1993) method based on preparative acid electrophoresis which allows Pin-b to be isolated from wheat flour in good yield and with a high degree of purity.

### MATERIALS AND METHODS

Seeds of soft wheat *Triticum aestivum* cv. Centauro harvested in 2000 were supplied from Società Produttori Sementi S.p.a. (Bologna, Italy) and stored in sealed glass containers at 10°C in the dark. The seeds were ground in a break roller mill (Labormill 4RB, Italy) and the flour was stored at -20°C.

Flour (130 g) was extracted at 4°C by vigorous stirring with 300 mL of 0.1M Tris-HCl (pH 7.8) containing 5 mM EDTA, 0.1M KCl, and 4% (w/v) Triton X-114. After centrifugation for 10 min (30°C, 11,000  $\times$  g) the supernatant was warmed at 30°C for 1 hr

and recentrifuged in the same operating conditions. The upper detergent-poor phase was discarded and replaced with an equal volume of fresh Tris-KCl buffer containing 0.06% Triton X-114. The solution was stirred for 1 hr at 4°C and the phase partition procedure was repeated. After centrifugation, the lower detergent-rich phase was mixed with 270 mL of a cold solution of diethylether and ethanol (1:3, v/v) and stored at -20°C overnight. The suspension was centrifuged for 10 min (10°C, 28,000  $\times$  g) and the pellet was washed five times with diethylether and ethanol (1:3, v/v) and once with diethylether. The suspension was centrifuged and the pellet was dried overnight at 20°C. The obtained powder was dissolved in 4 mL of 0.05M acetic acid. After centrifugation, solid NaCl was added to the supernatant (final concentration 2.0M). The suspension was centrifuged at 28,000  $\times$  g and the pellet was solubilized with 2 mL of 0.05M acetic acid, dialyzed overnight against 0.05M acetic acid and applied to a Sephadex G-50 column (2  $\times$  77 cm) eluted with the same solution. Fractions (1 mL) were collected and the elution was monitored on both the UV monitor (280 nm) and the chart recorder of the apparatus. Fractions from 90 to 150 mL were pooled, frozen, and lyophilized. The obtained powder (50 mg) was resuspended with 0.5 mL of 0.02M acetic acid containing 0.15 g of glycerol, and the sample was separated by continuous elution electrophoresis using a preparative apparatus (model 491 prep cell, Bio-Rad Laboratories, Richmond, CA). The gel composition for a length of 12 cm was 118.8 mg of ascorbic acid, 47.65 mL of water, 0.888 mL of acetic acid, 14.88 mL of ferrous sulphate (0.032%, w/v), 56.28 mL of acrylamide-bisacrylamide (15% T, 2.7% C), and 0.3 mL of hydrogen peroxide. The upper and lower chambers were filled with 0.02M and 0.04M acetic acid solutions, respectively. A runtime of 210 min at 400V and an elution flow rate of 60 mL/hr were employed. The elution was monitored on both the UV monitor (280 nm) and the chart recorder of the apparatus. Fractions of 2.5 mL were collected and analyzed by SDS-PAGE (Laemmli 1970). The fractions (26–37) containing the purified protein were pooled, frozen, and lyophilized. The presence of the puroindolines was monitored by a visual qualitative test performed on a membrane strip for the detection of nondurum wheat in pasta (Pastascan, Rhône Diagnostics, Glasgow, Scotland). The identity of the purified puroindoline was determined by N-terminal amino

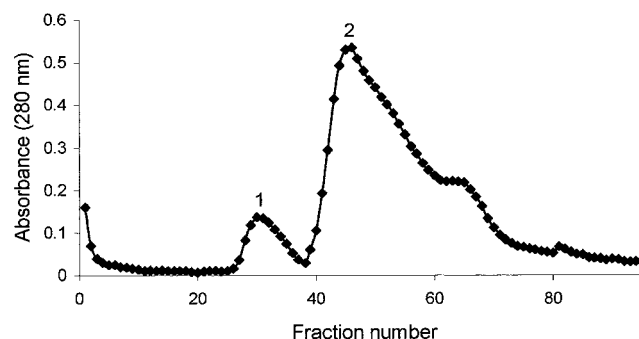
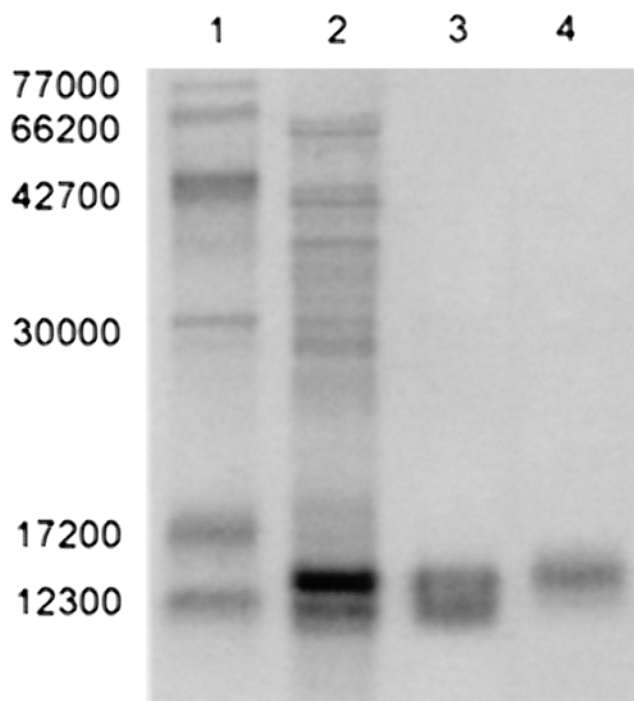


Fig. 1. Absorbance at 280 nm of protein fractions (2.5 mL) collected after preparative A-PAGE.

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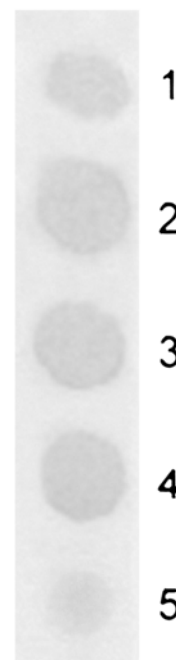
**Fig. 2.** SDS-PAGE (15% T; 2.7% C) under reduced conditions. Lane 1, molecular weight standards; lane 2, crude extract from soft wheat flour; lane 3, partially purified Pins after gel filtration; lane 4, purified Pin-b obtained after preparative A-PAGE (Peak 1). Gel was silver stained.

acids sequencing on a protein sequencer (MilliGen 6600, Millipore Corporation, Bedford, MA) according to the manufacturer's protocols.

## RESULTS AND DISCUSSION

We modified the method of Blochet et al (1993) to obtain pure Pin-b with a satisfactory yield. The modifications performed were based on the peculiar properties of Pins and mainly concerned the initial extraction and the final purification steps. In particular, the strongly alkaline isoelectric points reported for Pins suggested substituting cation-exchange chromatography, which is the last step in the method of Blochet et al (1993), with a preparative isoelectric focusing or an A-PAGE. The isoelectric focusing was performed with a preparative IEF cell (Rotofor, BioRad) and a buffer (pH 9.5–11.0) (RotoLyte, BioRad). However, the proteins loaded in the focusing chamber precipitated during the electrophoretic run, even if nonionic protein solubilizing agents were included, thus hampering the purification of Pins. Preparative A-PAGE proved to be an efficient and rapid technique to separate Pins, as demonstrated by Corona et al (2001), at analytical level. In particular, the spectrophotometric analysis of the collected fractions at 280 nm showed two peaks (Fig. 1). SDS-PAGE analysis revealed that peak 2 (fractions 39–80) is associated with a mixture of polypeptides (data not shown), whereas peak 1 (fractions 26–37) is due to a single protein. Figure 2 shows the electrophoretic patterns obtained at subsequent purification steps of this protein identified as Pin-b.

The presence of Pins in the partial and final products of the purification procedure was tested with the Pastascan kit, which contains a monoclonal antibody specific for friabilin and is routinely utilized for a rapid detection of nondurum wheat in durum wheat pasta. As shown in Fig. 3, the crude extract from soft wheat flour, the partially purified protein after gel filtration, and the purified protein after preparative A-PAGE showed a clear positive spot, revealing the presence of puroindolines, when compared with the nondurum pasta (positive control) and durum pasta (negative control) standards of the Pastascan kit. By using a similar test (Durotest P) Darlington et al (2000) demonstrated that the friabilin antibody



**Fig. 3.** Test kit membrane strip. Spot 1: 3% nondurum pasta standard of kit (positive control); spot 2: crude extract from soft wheat flour; spot 3: partially purified protein after gel filtration; spot 4: purified protein after preparative A-PAGE; spot 5: durum pasta standard of kit (negative control).

reacts positively with puroindolines from barley and wheat. However, the same authors showed that this antibody gives a positive reaction with both Pin-a and Pin-b. Thus, to define the purified protein as Pin-a or Pin-b, we determined its N-terminal sequence, which was EVGGGGGPQQCPQ. This sequence is identical to those previously reported in the literature for Pin-b, except for the eighth amino acid, which is a proline rather than a serine, as reported by Gautier et al (1994) and Lillemo (2001), or a glycine, as reported by Oda and Schofield (1997).

The method here described produced a satisfactory yield of purified Pin-b from flour (4 mg/130 g of flour). Thus, we can conclude that it is a suitable method for the isolation of high amounts of pure Pin-b to be used in physicochemical and technological studies.

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