

Yeast-derived potato patatins: Biochemical and biophysical characterization

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ABSTRACT

Potato patatin is considered a valuable plant protein by the food industry for its exceptional functional properties and nutritional value. Nonetheless, it has not been widely used due to its low abundance in potatoes and high cost. *Pichia pastoris* was utilized for expression of patatin to overcome agricultural limitations. Biochemical and biophysical characterization of Patatin-B2 (rPatB2) and Patatin-17 (rPat17) is described. rPatB2 and rPat17 had higher zeta potential and superior solubility at various pH conditions in comparison with commercial patatin, whereas particle size distribution was similar. Inflection temperatures were higher than potato isolated patatins. Antioxidant capacity of rPatB2 and rPat17 was similar to that of commercial patatin and the specific enzymatic activity of rPatB2 was 5-fold higher than rPat17 and patatins isolated from potato. Results indicate yeast-derived patatin properties are comparable to patatins from potatoes, suggesting their potential use in various plant-based products such as meat and dairy analogues.

1. Introduction

The rise in global population, and the subsequent increasing demand for an adequate supply of protein for food applications, is of current concern to government and industrial stakeholders. New protein sources for food applications are being considered ranging from insects and plants to recombinant proteins expressed in microorganisms (Grossmann & Weiss, 2021; Wegener, Jansen, & Jürgens, 2015). *Pichia pastoris* presents great potential as an alternative protein factory. Impossible Foods™ for example, utilize this system to produce soy leghemoglobin for their meat analog, the Impossible Burger (Waltz, 2019). Due to efficient secretion to the media, cell disruption is not needed to isolate the protein of interest, allowing for moderate purification methods which maintain protein functionality, reduce potential contaminants in the final product, without compromising recovery. Furthermore, concerns regarding toxins and antinutrients, such as the potato glycoalkaloids α -solanine and α -chaconine or protease inhibitors can be avoided by expression in *P. pastoris* (Løkra, Helland, Claussen, Stræt- kvern, & Egeland, 2008). Of the plant-based protein alternatives, potato proteins have gained great interest due to their superior essential amino acids composition compared to most plant based proteins and high biological value of 90–100 (Calero, Muñoz, Cox, Heuer, & Guerrero, 2013). In terms of functionality, they provide good emulsifying

property, excellent gel-forming capacity, and superb foamability and foam stability. Potato proteins are commonly obtained as a byproduct of the potato starch industry and can be divided into three groups: patatins, protease inhibitors and high molecular weight proteins (Kowalczyński et al., 2019).

Patatins, composing ~ 40% of soluble tuber proteins, are a groups of ~ 40 kDa glycoproteins most abundant in potato tuber (Pikkard, Brusca, Hannapel, & Park, 1987). Organ specific expression studies of the patatin multigene family classified two transcript classes. Class I patatin transcripts were exclusively found in potato tubers, while class II transcripts were found in both tubers and roots (Pikkard et al., 1987). Characterization of the 17 patatin gene groups during tuber development, revealed varying expression patterns of specific groups during the six developmental stages (Stupar et al., 2006). High variability in protein content and patatin isoform composition was recently shown in 15 different cultivates of *Solanum tuberosum* (*S. tuberosum*) (Bárta, Bártová, Zdráhal, & Šedo, 2012).

Unlike other storage plant proteins, patatin has been shown to possess enzymatic activity equivalent to a combination of different enzyme activities such as those of phospholipase A2, acyltransferase, glycolipase, esterase and nonspecific lipid acyl hydrolase. Recombinant expression of the isoenzyme Patatin-T5 in *Nicotiana tabacum* demonstrated esterase activity, while site-directed mutagenesis revealed that

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glycosylation is not essential for activity nor does it affect substrate specificity (Sonnewald, Von Schaewen, & Willmitzer, 1990). The class I patatin, Patatin-B2, was first isolated from *S. tuberosum* Bintje cultivar tubers (Stiekema et al., 1988). Recombinant Patatin-B2, refolded and purified from *E. coli* inclusion bodies, possessed esterase activity on esters with varying chain-lengths. Site-directed mutagenesis confirmed that Patatin-B2 is a serine hydrolase in which Ser54 and Asp192 form the catalytic dyad (Hirschberg, Simons, Dekker, & Egmond, 2001). Further confirmation of this mechanism was later reported for the Patatin-17 isoenzyme from *Solanum cardiophyllum*, expressed in *P. pastoris*, which shares 90% and 87% sequence identity with Patatin-T5 and Patatin-B2, respectively (Rydel et al., 2003).

The main hypothesis of the current study is that a recombinant patatin produced by fermentation from a food grade yeast will be devoid of antinutritional and toxic elements and may have somewhat different functional properties than commercial patatin purified from potatoes and comprising several isoenzymes. Although recombinant patatins have been used to characterize the catalytic mechanism and protein structure, a biochemical and biophysical comparison of patatin isolated from potatoes and recombinantly expressed, has not been reported so far. Our comparative study reveals that recombinant patatins expressed in *P. pastoris* have equal or even superior characteristics compared to patatin isolated from potatoes in most tested parameters. This implies that recombinant patatins, expressed and secreted from *P. pastoris*, could have great potential as an alternative protein source for food applications, such as meat and dairy analogues, or other plant-based products.

2. Material and methods

2.1. Materials

Unless otherwise specified, chemicals and reagents were purchased from Sigma (Rehovot, Israel). Restriction enzymes were from New England Biolabs (Ipswich, MA, U.S.A.). Zeocin was from InvivoGen (San Diego, CA, U.S.A.). Coomassie brilliant blue (R-250) and Bradford reagent were purchased from BioRad (Rishon Le Zion, Israel). *Pichia pastoris* strain X-33 was kindly provided by Prof. Niv Papo, Ben-Gurion University. Peptone, yeast extract, yeast nitrogen base and agar were from Becton, Dickinson and company (Franklin Lakes, NJ, U.S.A.). Glycerol, triton X-100, sodium chloride and sodium hydroxide were purchased from BioLab (Jerusalem, Israel). Sorbitol was purchased from Alfa-Aesar (Ward Hill, MA, U.S.A.). Imidazole was purchased from spectrum (Gardena, CA, U.S.A.). Sucrose was purchased from J.T.Baker (Phillipsburg, NJ, U.S.A.). Ethanol and methanol were purchased from Gadot-group (Netanya, Israel). NucleoBond Xtra Midi was purchased from MACHEREY-NAGEL (Düren, Germany). DMSO (dimethyl sulfoxide) and acetic acid were purchased from Carlo Erba (Barcelona, Spain). *p*-Nitrophenyl hexanoate was purchased from TCI (Tokyo, Japan). Nitrocellulose membranes were purchased from PALL life sciences (New York, U.S.A.). The commercial Solanin® potato protein 200 was obtained from Avebe (Avebe A.U., Foxhol, Netherlands). IEF gels and buffers were purchased from Invitrogen by Thermo Fisher Scientific (Carlsbad, CA, U.S.A.).

2.2. Cloning of patatin in *P. Pastoris*

Solanum tuberosum Patatin-B2 (accession number: P15477) and *Solanum cardiophyllum* Patatin-17 (accession number: Q8LPW4) genes with codon optimization for *Pichia pastoris* and C-terminal 6xHistidine tag were purchased from Twist Bioscience. The genes were cloned, without their endogenous signal peptide, into pPICZα between *Xho*I and *Not*I, transformed into *E. coli* DH5α and selected on low-salt LB plates with 50 µg/mL Zeocin (InvivoGen). Open reading frame, with the yeast secretion signal peptide, was confirmed by sequencing.

For gene transformation to *P. pastoris*, 250 mL of *E. coli* DH5α were grown overnight at 37 °C in 250 mL of LB medium with 50 µg/mL Zeocin

and plasmids were extracted by MidiPrep (NucleoBond Xtra Midi, MACHEREY-NAGEL). 100 µg of each plasmid was linearized overnight at 37 °C, pPICZα-PatB2 with *Pme*I and pPICZα-Pat17 with *Sac*I-HF (New England Biolabs, Ipswich, MA, U.S.A.), followed by ethanol precipitation and resuspension in 15 µL molecular biology grade water. Electrocompetent *P. pastoris* X-33 were prepared according to the Pichia EasyComp™ Kit protocol (Invitrogen, CA, U.S.A.). Transformants were selected on YPDS plates (2% peptone, 1% yeast extract, 2% D-glucose, 18.2% sorbitol and 2% agar) containing 500 µg/mL Zeocin, at 30 °C for 3 days. For clone selection, six colonies of each transformation were picked into 5 mL of BMGY medium (2% peptone, 1% yeast extract, 0.23% K₂HPO₄, 1.181% KH₂PO₄, 1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin and 1% glycerol) and grown 24 h at 30 °C with vigorous shaking. The next day, 5 mL of BMMY medium (2% peptone, 1% yeast extract, 0.23% K₂HPO₄, 1.181% KH₂PO₄, 1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin and 0.5% methanol) were inoculated with 0.5 mL BMGY culture, for 72 h of protein expression. 0.5% methanol was added every 24 h, to maintain expression induction. Cell and media of the different clones were run on SDS-PAGE and protein expression and secretion were evaluated by SDS-PAGE.

2.3. Patatin protein production

The highest expressing clone of each gene was used for large scale protein expression and purification. Fifty mL of BMGY media were inoculated from the glycerol stock and grown for 24 h at 30 °C with vigorous shaking. The next day, 500 mL of BMMY media were inoculated with the entire BMGY culture for 9 days of protein expression at 30 °C. Methanol was added daily to maintain expression induction (0.5% for rPatB2 and 1.0% for rPat17). After 9 days, cells were separated from media by centrifugation (4,000 × g, 4 °C, 10 min). Cleared media was supplemented with 500 mM NaCl and 20 mM imidazole, pH was adjusted to pH 8.0 and the media was incubated for 1 h at 4 °C. Precipitation was removed by centrifugation (16,000 × g, 4 °C, 20 min) and supernatant was loaded on a 5 mL HisTrap HP prepacked nickel affinity column (GE Healthcare Bio-Sciences AB), equilibrated with 20 mM Tris-HCl pH 8.0, 500 mM NaCl and 20 mM imidazole, using an AKTA prime plus FPLC system (GE Healthcare Bio-Sciences AB). Protein was eluted from the column with 20 mM Tris-HCl pH 8.0, 500 mM NaCl and 500 mM imidazole, and peak fraction dialyzed overnight against 10 mM Tris-HCl pH 8.5 (12 kDa cutoff). Protein concentrations were determined by either Bradford assay (for load and flow-through samples) or absorbance at 280 nm for purified protein, in a TAKE3 plate using Synergy H1 microplate reader (BioTek). Extinction coefficient and molecular weight for PatB2 were 34,840 M⁻¹cm⁻¹ and 40.95 kDa and for Pat17 36,330 M⁻¹cm⁻¹ and 40.82 kDa. Protein purity was evaluated by SDS-PAGE Coomassie staining.

2.4. Sodium dodecyl sulfoxide – Poly acrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as previously described (Laemmli, 1970) with 4% acrylamide in the stacking gel and 12% in the resolving gel. Before loading, samples were boiled for 10 min at 100 °C in reducing sample buffer. To evaluate protein purity, gels were stained with 0.25% Coomassie brilliant blue (R-250), 50% ethanol & 10% acetic acid followed by de-staining with 20% methanol and 10% acetic acid. Protein identity was verified by Western blot. Gels were transferred to nitrocellulose membranes (PALL life sciences) in 25 mM Tris, 193 mM glycine & 10% methanol for 2 h at 220 mA in ice. Membranes were blocked with 3% bovine serum albumin (BSA) (Sigma) in TBST (20 mM Tris-HCl pH 7.5, 137 mM NaCl & 0.1% Tween-20) for 2 h at room temperature followed by blotting for an additional 2 h with mouse anti His-tag HRP (R&D systems). Finally, bands were detected using Immobilon Crescendo Western HRP chemiluminescence substrate from Merck Millipore (Temecula, CA, USA) and visualized in FUSION FX imaging system

(Vilber).

Isoelectric focusing was performed on a 3–7 IEF gel (Invitrogen). Gels were run for 1 h at 100 V, 1 h at 200 V and 1.5 h at 500 V and fixed in 12% TCA before being stained with Coomassie brilliant blue (R-250) as described for SDS-PAGE. Liquid mix IEF markers 3–10 (Serva) was used to estimate the isoelectric point.

2.5. Commercial patatin stock preparation

0.25 g Solanic 200 (Solanica® Avebe) was dissolved in 50 mL 10 mM Tris-HCl pH 8.5 for 1 h at room temperature. Insoluble particles were removed by centrifugation ($4,000 \times g$, 10 min) and protein concentration in the supernatant was determined by Bradford Protein assay (Bio-Rad) with a BSA calibration curve.

2.6. Potato protein isolate preparation

Potato Protein Isolate (PPI) was prepared as previously described (Glusac, Isaschar-Ovdat, Kukavica, & Fishman, 2017). Working stock solution was prepared using lyophilized protein as described in section 2.5.

2.7. Esterase activity assay

Catalytic activity based on the hydrolysis of *p*-nitrophenyl octanoate (pNP-Oct) was performed in 96-well plates as previously described by Rydel et al. with minor modifications (Rydel et al., 2003). Stock solution of pNP-Oct was prepared in DMSO (20 mM) and diluted 5-fold in 4% Triton X-100 and 1% SDS to 4 mM. The substrate was then further diluted 20-fold for the reaction solution in 50 mM Tris-HCl pH 8.5 & 1 mM CaCl_2 to a final concentration of 0.2 mM. For a standard assay, 180 μL of reaction solution was pre-equilibrated to 30 °C for 5 min, followed by addition of 20 μL diluted enzyme and reaction was monitored in a microplate reader at 405 nm for 10 min with 35 s intervals. For data analysis, absorbance at 405 nm was converted to pNP concentration (mM). One enzymatic unit was defined as the amount of enzyme that will release 1 μmole of pNP per minute under these assay conditions. Unit concentration was converted to specific activity by dividing by the protein concentration.

2.8. Substrate specificity

For substrate specificity, stock solutions of 20 mM were prepared in DMSO for six different substrates: pNP-Butyrate, pNP-Hexanoate, pNP-Octanoate, pNP-Decanoate, pNP-Dodecanoate and pNP-Palmitate, and diluted as described above in the catalytic activity assay section to 1 mM. To compensate for differences in specific activity, protein concentrations in the assay were: 0.5 $\mu\text{g/mL}$ rPatB2, 2.5 $\mu\text{g/mL}$ rPat17 and 3.5 $\mu\text{g/mL}$ Solanic 200 or PPI.

2.9. pH solubility and stability

pH solubility was measured as previously described (Waglay, Karboune, & Alli, 2014). In brief, protein samples of 5 mg/mL were diluted 5-fold in 125 mM buffer (KCl-HCl pH 2.0, sodium acetate pH 3.0–5.0 and sodium phosphate pH 6.0–9.0), giving final assay conditions of 1 mg/mL protein and 100 mM buffer. Samples were incubated for 30 min at room temperature, and insoluble particles were removed by centrifugation ($4,000 \times g$, 10 min, RT). Soluble protein concentration was determined according to the Bradford Protein assay. Residual activity of the soluble protein was measured by pNP-Oct hydrolysis as described above for the esterase activity assay. For both assays, samples were diluted in 50 mM Tris-HCl pH 7.5 and 100 mM NaCl, to minimize effect of pH and ionic strength on assay conditions.

2.10. Temperature solubility and stability

Temperature solubility was measured by incubating protein samples of 1 mg/mL in 10 mM Tris-HCl pH 8.5 at 30–100 °C for 1 h. Insoluble particles were removed by centrifugation ($4,000 \times g$, 10 min, RT). Soluble protein concentration and residual enzymatic activity were measured as described above for pH solubility.

2.11. Thermal-induced unfolding

Thermal-induced unfolding was measured by differential scanning calorimetry (DSC 250, TA Instruments, New Castle, DE, USA) as previously described (Schmidt et al., 2019) with some changes. Samples of 5 mg protein dispersion (30% w/v in Tris buffer 0.5 M) were weighed and sealed in hermetically aluminum pans and heated from 25 to 100 °C at a rate of 1 °C/min under nitrogen at a flow rate of 50 mL/min. Denaturation temperature and enthalpy were calculated from the thermograms using TRIOS software (TA Instruments). Duplicate measurements were performed for each sample.

Thermal-induced unfolding was also determined by changes in tryptophan fluorescence at emission wavelengths 330 nm and 350 nm as described by (Singh et al., 2019). Protein samples of 5 mg/mL in dialysis buffer were loaded into UV capillaries and fluorescence was monitored using Tycho NT.6 (NanoTemper Technologies, Germany) from 35 °C to 95 °C at a rate of 30 °C per minute. Inflection temperature was calculated by the device. Samples were measured in duplicate.

2.12. Particle size distribution and zeta potential

Particle size distribution (PSD) and ζ -potential of the samples were determined using Zetasizer Ultra (Malvern Instruments, Worcestershire, UK). Particle size distribution was determined by dynamic light scattering as previously described (Ruseva et al., 2018) with some changes. One mL of 0.5 mg/mL protein sample in 10 mM Tris-HCl pH 8.5 was transferred to a 10 mm cuvette. The instrument was equilibrated for 2 min and measurements were taken at 25 °C. ζ -potential of the samples was measured at three pH conditions: 10 mM sodium acetate pH 3.0, 10 mM sodium phosphate pH 6.0, and 10 mM Tris-HCl pH 9.0. Measurements were performed as previously described by (Pascoviche, Goldstein, Fishman, & Lesmes, 2019) with small changes. Protein samples of 5 mg/mL were diluted 8-fold in appropriate buffer and transferred to the test cuvette. The instrument was equilibrated for 2 min, followed by at least 10 readings per sample within a measurement. The electric charge of the proteins was calculated by the device software using the Smoluchowski model.

Each sample preparation was measured in triplicate and 3 independent sample preparations were tested for PSD and ζ -potential.

2.13. Antioxidant capacity

Antioxidant capacity was determined using the Oxygen-Radical Absorbance Capacity (ORAC) assay in 96-well black microtiter plates as previously described (Francini et al., 2017). Protein samples were 1 mg/mL and Trolox calibration curve 10–50 μM in dialysis buffer (10 mM Tris-HCl pH 8.5). For a standard assay, 150 μL of 8 nM fluorescein, in 75 mM phosphate pH 7.4, were equilibrated for 30 min at 37 °C with 25 μL standards or protein samples. Upon addition of 25 μL 75 mM 2,2'-Azobis (2-methylpropionamide) dihydrochloride (AAPH), fluorescence was measured in Synergy H1 microplate reader for 2 h at 37 °C with 1-min intervals using excitation and emission wavelengths of 485 nm and 535 nm, respectively. For each well, fluorescence at each time point was normalized to fluorescence at time zero. Area under the fluorescence decay curve (AUC) of blank wells were subtracted from the samples (net AUC). A calibration curve of net AUC vs Trolox concentration was prepared from which equivalent Trolox concentrations in the samples were calculated. Antioxidant capacity expressed as Trolox amount per

gram protein by dividing Trolox concentration by protein concentration.

2.14. Circular dichroism (CD) spectra

Circular dichroism (CD) spectra analysis was performed according to Pots et al. with slight changes (Pots, De Jongh, Gruppen, Hessing, & Voragen, 1998). Stock solutions of 0.1 mg protein/mL were prepared in distilled water. Samples were centrifuged at 5,000 g for 5 min to remove insoluble particles. Two and a half milliliters of stock solution were transferred to a 3 mL quartz cuvette with an optical path length of 1 cm. CD spectra were scanned at 25 °C on a Jasco-1100 spectrophotometer (JASCO, Easton, MD) between 190 and 260 nm with 1 nm intervals and scan speed of 100 nm/min, and recorded as average of three spectra. Blank signal was subtracted to correct the spectra of protein samples and the resultant spectra were deconvoluted and analyzed using CDNN 2.1 software (Applied Photo - physics Ltd., Surrey, UK).

2.15. Absorbance and intrinsic fluorescence spectra

Protein absorbance was measured in Varioskan Flash plate reader (Thermo Scientific, Finland) using a 96-well quartz plate. Stock solutions of 1.0 mg protein/mL buffer (10 mM Tris-HCl pH8.5) were centrifuged at 5,000 g for 5 min, 200 µL were transferred to the quartz plate and spectra was measured from 200 nm to 600 nm. For intrinsic fluorescence, excitation was set to 280 nm and emission was measured between 300 nm and 400 nm.

2.16. Hydrophobic surface

The hydrophobic surface of Patatin-17 and Patatin-B2 was visualized using Chimera 1.14. The structure of Patatin-17 was solved by Rydel et al., therefore PDB 4PK9 was used for visualization (Rydel et al., 2003). The structure of Patatin-B2 has not been solved, and so a model was prepared. The full sequence of Patatin-B2 (accession number: P15477) was uploaded to the SWISS-Model server (<https://swissmodel.expasy.org/>), followed by a search for templates. The template which had the highest score and identity (PDB 4PKA) was selected as the basis for the model.

2.17. Proteomic analysis

Bands of rPatB2 were cut from acrylamide gel, subjected to chymotrypsin digestion, analyzed by LC-MS/MS on Q-Exactive plus mass spectrometer (QE, Thermo Fisher Scientific™, Waltham, MA USA), and identified with Protein Discoverer 1.4 software using the Sequest algorithm (Thermo Fisher Scientific™, Waltham, MA USA) against the pPICZα-PatB2 sequence and *P. pastoris* database.

2.18. Statistics

All data were subjected to one-way analysis of variance (ANOVA), except for zeta potential for which two-way ANOVA was carried out to assess the difference in means between proteins within each pH condition and between pH conditions for every protein, and compared by the Tukey's HSD test (SigmaPlot 14.5, Systat Software, Inc., USA). The level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Single-step purification of recombinant patatin

Expression levels of recombinant proteins in *P. pastoris* are protein dependent. Expression of human IL-25 and phospholipase A2 in *P. pastoris* produced 100 and 125 mg/L, respectively (Filkin et al., 2020; Liu, Wu, Wang, Mo, & Yu, 2013). Meanwhile, expression of *Trichoderma reesei* tyrosinase 2 in this system was only 24 mg/L after optimization

and human ADAM-9 produced a mere 0.1 mg from one liter of culture (Schwettmann & Tschesche, 2001; Westerholm-Parvinen et al., 2007). *P. pastoris* has been used for expressing Pat17, however no information regarding yield was reported (Rydel et al., 2003). In this study, a typical experiment employing 500 mL growth media in shake flasks, resulted in 100–200 mg of rPatB2 and 20–60 mg rPat17. Several isolation methods have been developed over the years for extraction of potato proteins, including acidic, thermal and ammonium sulfate precipitation (Waglay et al., 2014). While some of these methods provide high protein yield, this is often accompanied by loss of functionality. In recent years, gentler methods have been utilized to preserve functionality of purified potato protein, such as expanded bed adsorption chromatography employed by Avebe for the Solanic 200 product (Løkra et al., 2008). Incorporation of a C-terminal hexa-Histidine tag to recombinant Patatin-B2 (rPatB2) and Patatin-17 (rPat17), enabled the use of a nickel affinity column. The single-step purification produced fully functioning proteins with high yield and excellent purity. A typical purification table of the recombinant proteins is presented in Table 1. Expression level of rPatB2 was higher than of rPat17, however, purification of rPat17 yielded 100% of the expressed protein while purification of rPatB2 yielded only ~80%. Precipitation in the rPatB2 sample during application on the affinity column, possibly due to its higher concentration in the media, could account for its lower yield. SDS-PAGE Coomassie staining did not show presence of rPatB2 in the flow-through, suggesting the lower yield did not result from insufficient binding to the affinity column (Fig. S1).

3.2. Esterase activity and substrate specificity

Esterase activity based on pNP-Oct hydrolysis showed that the specific activity of purified rPatB2 was ~5-fold higher than that of purified rPat17 (Table 1) and ~2.5-fold higher than Solanic 200 and PPI (data not shown). Wu et al. recently showed that patatins isolated from *S. tuberosum* possess anti-obesity effects in zebrafish, and suggested this could have been achieved via affecting lipid metabolism (Wu et al., 2021). Thus, the higher specific activity of rPatB2 could make it a good candidate for evaluation of its anti-obesity properties in other animals and humans.

Substrate specificity for patatins esterase activity was measured on *p*-nitrophenyl-esters with different chain lengths (Fig. 1). Although pNP-Oct was used as a substrate when determining esterase specific activity (Rydel et al., 2003), the highest relative activity for all patatins was on pNP-Decanoate (C10) and all values were normalized to its activity. The lowest relative activity of all four proteins was on the shortest substrate, butyrate. Although rPatB2 and rPat17 share over 85% sequence identity (Fig. S2), their relative activity on different substrates was slightly different. The specificity pattern of rPatB2 was similar to the specificity of Solanic 200 and PPI, while rPat17 had higher relative activity on pNP-Hexanoate, pNP-Octanoate and pNP-Palmitate substrates. Previous work on recombinant patatin, expressed in *E. coli* and refolded from inclusion bodies, showed a specificity pattern resembling rPat17 (Hirschberg et al., 2001). These results suggest that different patatin isoenzymes possess distinct substrate preferences.

3.3. Particle size distribution and protein profile

Particle size distribution of rPatB2 and rPat17 was compared to commercial Solanic 200 patatin and PPI and analysis revealed that the four protein samples share similar diameter of 6–9 nm (Fig. 2A). Protein profile and purity of recombinant and endogenously expressed patatins were evaluated by SDS-PAGE Coomassie staining (Fig. 2B). Patatins of both Solanic 200 and PPI ran at the expected 40–43 kDa (Bárta et al., 2012) while rPat17 migrated slightly above them in the gel and rPatB2 even higher, at 45 kDa. The increased molecular weight of the recombinant proteins could result from glycosylation pattern variability between potato and yeast (Sonnewald, Sturm, Chrispeels, & Willmitzer, 1989). Furthermore, Patatin-B2 has two predicted glycosylation sites

Table 1

A typical recombinant patatin purification table.

Sample		Total protein (mg)	Total Activity (U) ³	Specific activity (U/mg)	Yield (%)	Purification factor
rPatB2	Load	434.0 ¹	3805.3	8.8	100.0	1.0
	Flow-through	155.0 ¹	15.5	0.1	0.4	0.0
	Elution	122.2 ²	3172.6	26.0	83.4	3.0
rPat17	Load	171.3 ¹	191.0	1.1	100.0	1.0
	Flow-through	131.3 ¹	9.3	0.1	4.9	0.1
	Elution	42.6 ²	191.3	4.5	100.2	4.0

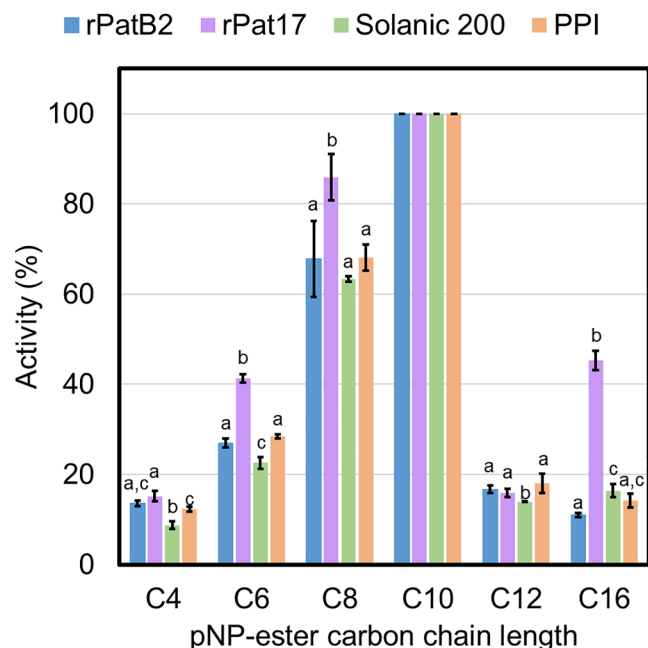
¹ Quantitated by Bradford assay.² Quantitated by absorbance at 280 nm with extinction coefficient and molecular weight – 34,840 M⁻¹cm⁻¹ and 40.95 kDa for rPatB2 and 36,330 M⁻¹cm⁻¹ and 40.82 kDa for rPat17.³ One unit (U) is defined as the amount of enzyme that releases 1 μmole of pNP per minute under assay conditions.

Fig. 1. Substrate specificity on *p*-nitrophenyl esters of different chain lengths. All values were normalized to activity with pNP-Decanoate. Blue – recombinant Patatin-B2, purple – recombinant Patatin-17, green – Solanic 200 and orange – potato protein isolate. Letters represent statistically different ($P < 0.05$) results between proteins within each carbon chain length. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(N115 and N325) while Patatin-17 has only one predicted site (N202) (Bateman et al., 2021). As glycosylation increases molecular weight of proteins, this could account for the observed migration differences in the gel between the recombinant proteins. Proteomic analysis confirmed the identity of the upper smear above the main band of rPatB2 to be Patatin-B2 rather than an impurity (top panel in Fig. 3). PPI has several low molecular bands between 15 and 20 kDa, previously identified as protease inhibitors (Glusac et al., 2017). The lower molecular weight bands in rPatB2 were previously shown when Patatin-B2 was expressed in *E. coli* (Hirschberg et al., 2001). These bands were not observed when rPatB2 and rPat17 were run without boiling (Fig. 2C). Absence of the lower molecular weight bands in the recombinant proteins when samples were not boiled, suggests that, unlike the additional bands in Solanic 200 and PPI, these bands may be an artifact of sample boiling rather than impurities. Proteomic analysis of the lower molecular bands observed in SDS-PAGE confirmed these were fragments of rPatB2 (bottom panel in Fig. 3 shows the coverage obtained). Although Solanic 200 is described by Avebe to be a native protein, sample boiling before SDS-PAGE analysis did not affect band migration in the gel, implying it might

be at least partially denatured. As Solanic 200 preparation is provided as a spray dried powder, it is possible that although purified in its native form, the high temperature used during spray drying (Løkra et al., 2008) caused partial protein denaturation.

3.4. Protein isoelectric point and surface charge

The isoelectric point of patatin has been reported to be between 4.5 and 5.2 (Pots, Gruppen, Hessing, Van Boekel, & Voragen, 1999). Bárta et al. compared 20 potato cultivars and found high variability in the number of isoelectric bands; from as few as seven, up to as many as 14 bands (Bárta et al., 2012). Höfgen compared isoelectric focusing (IEF) patterns of crude potato extracts from Berlina and Desire *S. tuberosum* cultivars, known to possess differences in patatin content (Höfgen & Willmitzer, 1990). While proteins of the Desire extract seemed to have very few isoelectric forms ranging from 4.9 to 5.5, the Berlina extract presented proteins with a wide variety of isoelectric points between 4.7 and 5.2 (Höfgen & Willmitzer, 1990). Isoelectric focusing of recombinant and potato isolated patatins is presented in Fig. 4A. The PPI pattern resembles the pattern shown for the Berlina extract. The isoelectric pattern of Solanic 200 proteins was more heterogenic, from 4.4 to 5.5. In both cases the pattern tended to be blurry, possibly due to high protein versatility. Meanwhile, both rPatB2 and rPat17 presented distinct bands, however, bands of rPat17 migrated farther than bands of rPatB2, implying a lower isoelectric point. The sequence based calculated isoelectric points of rPatB2 and rPat17 are 5.78 and 5.25, respectively, supporting a more acidic isoelectric point for rPat17. However, sequence-based calculations do not consider protein structure and post-translational modifications which may alter the isoelectric point (Zhu, Zhao, Lubman, Miller, & Barder, 2005). As rPatB2 is predicted to have two glycosylation sites while rPat17 is predicted to have only one site, an additional glycosylation site could account for the pattern difference between the two recombinant proteins. Although a variety of bands was observed in both recombinant proteins, these were reproducible between independent batches of the recombinant proteins (data not shown).

An important characteristic of potential food components is their zeta potential, as it can affect interactions with other components influencing food properties. For example, studies of an Iranian yoghurt stabilized with tragacanth gum, involved changes in zeta potential that inhibited casein aggregation, and cold-induced gelation studies of α -lactalbumin and β -lactoglobulin reported that acidification from pH 7.5 to pH 5.4 facilitated gelation by increasing the zeta potential (Cano-Sarmiento et al., 2018). Zeta potential at three pH conditions is presented in Fig. 4B. rPatB2 had the highest positive zeta potential at acidic pH (pH 3.0), $+16.2 \pm 0.6$ mV, but no differences were observed in zeta potential of rPat17, Solanic 200 and PPI in this environment. At close to neutral conditions (pH 6.0), above the isoelectric point, net negative surface charge of both rPatB2 and rPat17 was significantly higher than Solanic 200 and PPI, -18.8 ± 4.4 mV and -17.0 ± 1.3 mV vs -12.0 ± 2.2 mV and -9.0 ± 0.8 mV, respectively. Net negative surface charge of

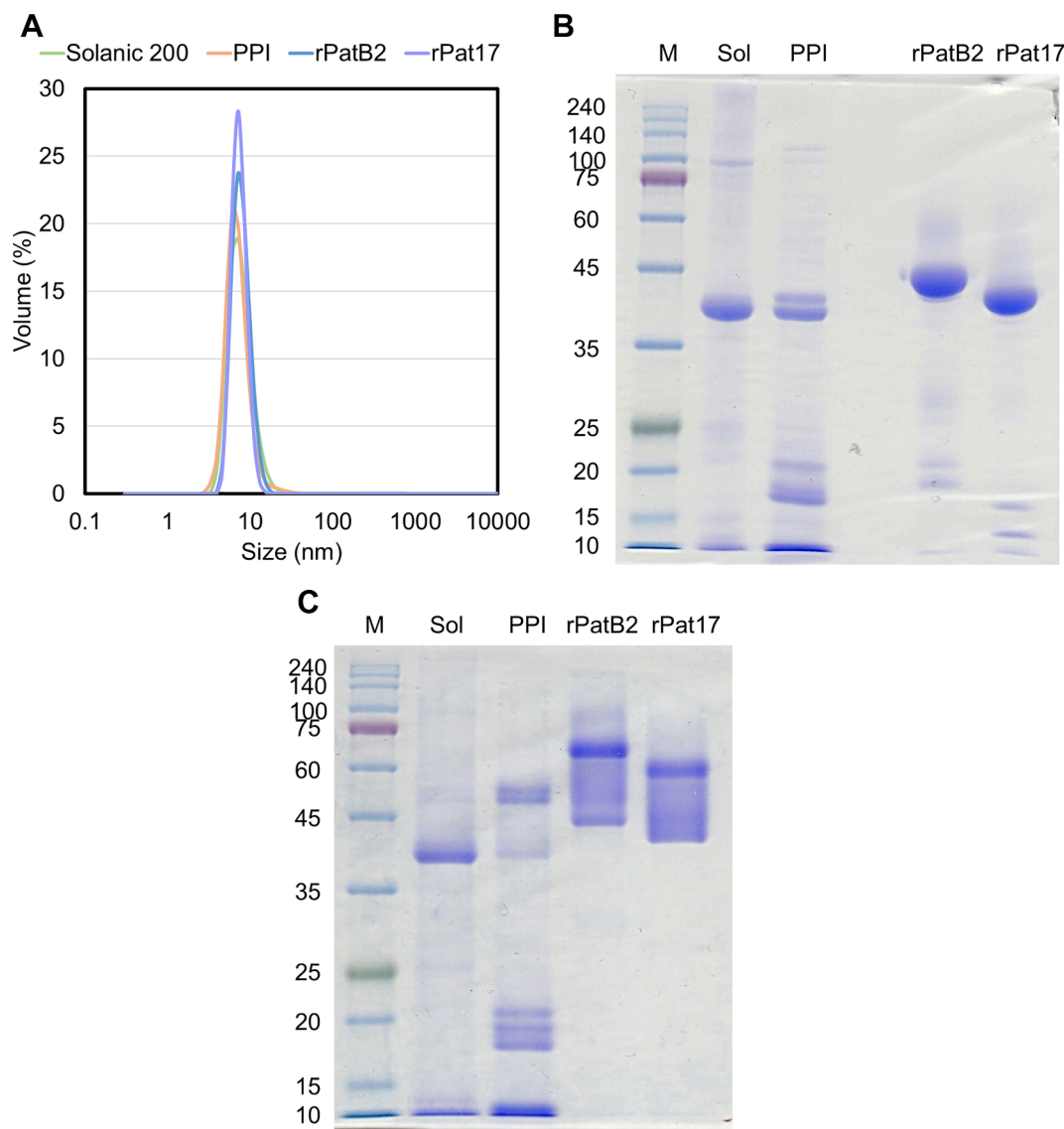


Fig. 2. Particle size distribution (A) and purity (B and C) of recombinant and endogenously expressed patatins. A. particle size distribution by dynamic light scattering, B. and C. SDS-PAGE with (B) or without (C) sample boiling. In B and C: Sol – Solanic 200, PPI – potato protein isolate, rPatB2 – recombinant Patatin-B2 and rPat-17 – recombinant Patatin-17. Gel: 12% acrylamide, Load: 10 μ g by Bradford.

the two recombinant proteins at pH 6.0 are comparable with those previously reported for chickpea, faba bean, lentil, pea and soy protein isolates (Karaca, Low, & Nickerson, 2011). The zeta potential measured for Solanic 200 at pH 6.0 is similar to those previously reported for patatin isolated from potatoes at pH 7.0 (Schmidt, Damgaard, Greve-Poulsen, Larsen, & Hammershøj, 2018). As expected, more negative charge of the recombinant proteins was observed at alkaline conditions (pH 9.0), at which surface charge of all four samples was $\sim$ 50% higher than at neutral conditions. Chang et al. showed an increase in zeta potential of PPI from -1.9 mV to -17.8 mV when dispersant conditions were changed from pH 3 to pH 6, and concluded that the pI of PPI is at pH 3.3 (Chang et al., 2020). We obtained similar values at pH 3.0 when the protein samples were added to the dispersant within the cuvette; this was followed by protein precipitation observations at the end of measurement (data not shown). However, when the protein sample was mixed with the dispersant prior to being transferred to the cuvette, the solution remained clear, zeta potential of PPI was $+10$ mV and the isoelectric point was estimated close to pH 4.5, as potential at pH 6.0 was -9 mV (Fig. 4B). Both the IEF gel presented in Fig. 4A and previous studies of patatin from potato showed isoelectric points between 4.6 and

5.2 (Pots et al., 1999). We hypothesized that injecting a small volume of concentrated protein sample in alkaline buffer (pH 8.5) into the acidic dispersant created a microenvironment around the protein of pH close to its isoelectric point, causing its precipitation. In which case, the zeta-potential measured did not truly represent its surface charge under acidic conditions.

3.5. Solubility and stability

Solubility is an essential functional property of proteins for food applications. Characterizing protein solubility under various conditions can facilitate identification of proteins soluble under unique conditions; this in turn may enable future incorporation of these proteins as ingredients in food products which possess such conditions. Solubility and residual activities measured in a wide range of pH (pH 2.0–9.0) or temperature (30–100 $^{\circ}$ C) conditions are presented in Fig. 5. Both recombinant proteins as well as PPI remained completely soluble across all tested pH conditions (Fig. 5A). In contrast, solubility of Solanic 200 at pH 5.0 was only $\sim$ 60%. Minimal solubility at pH 5.0 was previously shown for the patatin fraction of PPI (Schmidt et al., 2019). As patatin

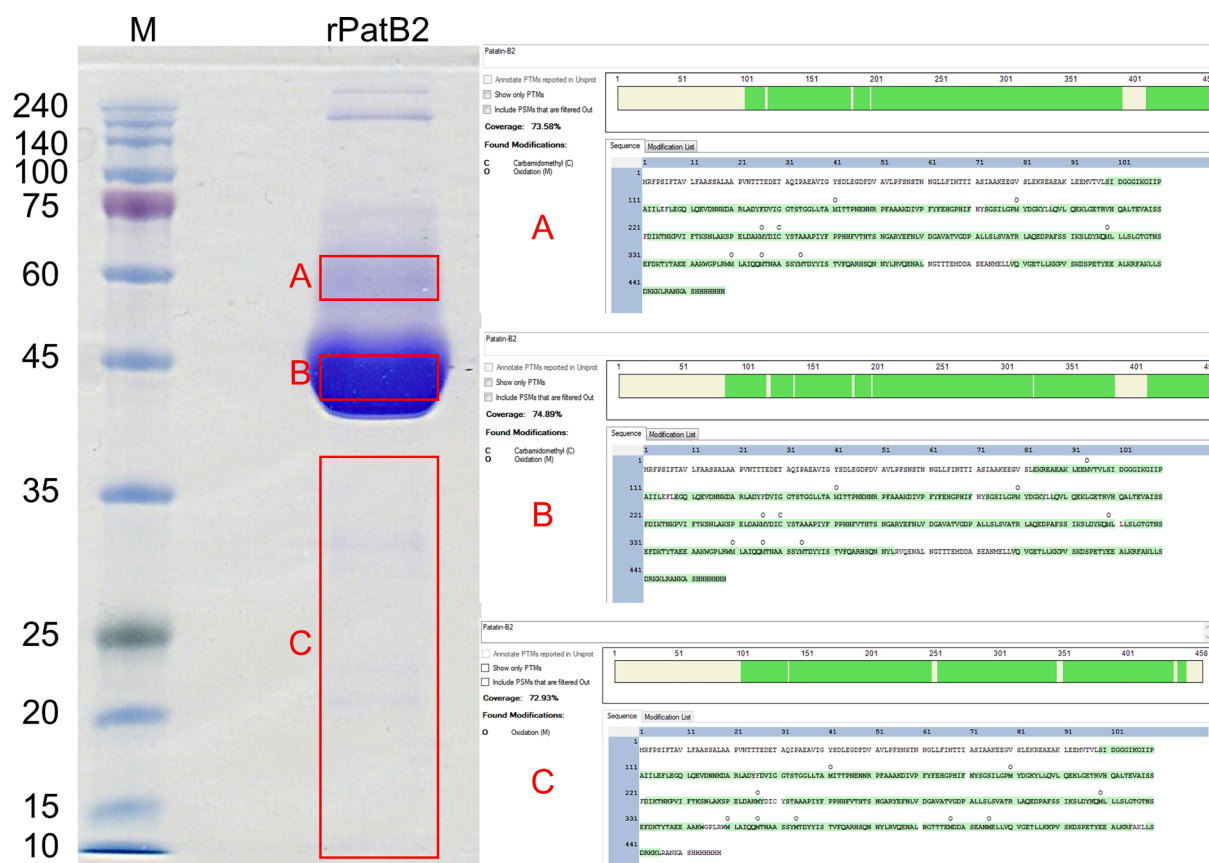


Fig. 3. Proteomics analysis of rPatB2 SDS-PAGE bands. Green background indicates amino acids identified in rPatB2 peptides produced by chymotrypsin digestion and detected by mass-spectrometry. Rectangles on the gel show the bands analyzed by LC-MS/MS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

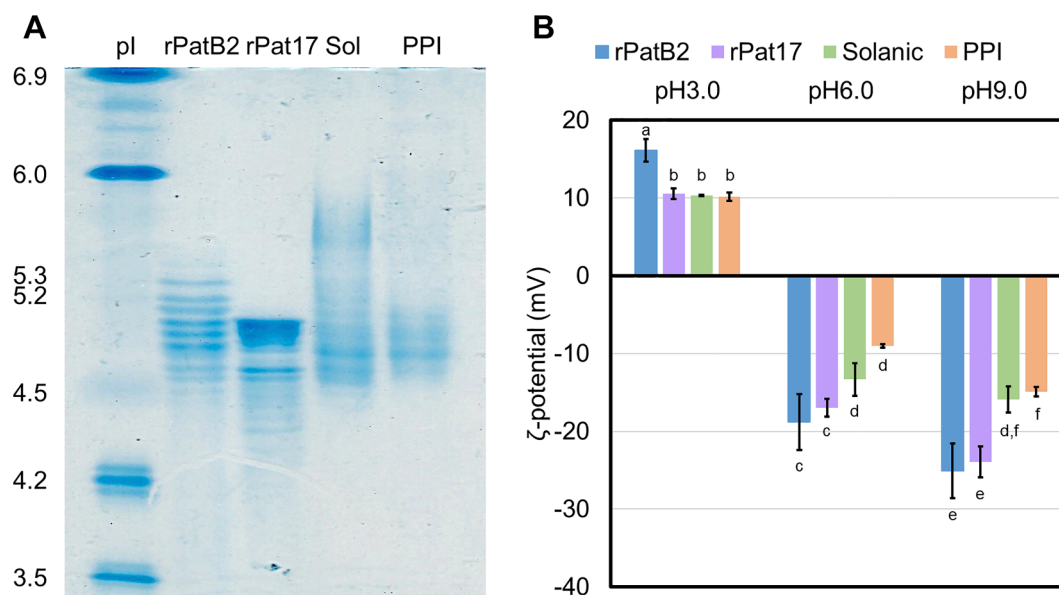


Fig. 4. Isoelectric focusing (A) and zeta-potential (B) of recombinant and endogenously expressed patatins. A: Gel: 3–7 IEF gel, Load: 10 µg by Bradford. B: pH3.0 – 10 mM Acetate pH 3.0, pH 6.0 – 10 mM Phosphate pH 6.0, pH 9.0 – 10 mM Tris-HCl pH 9.0, blue – recombinant Patatin-B2, purple – recombinant Patatin-17, green – Solanic 200 and orange – potato protein isolate. Letters represent statistically different ($P < 0.05$) results between proteins within each pH condition and between pH conditions for every protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was found to possess lipid acyl hydrolase activity (Hirschberg et al., 2001), relative activity was used as a measure for protein stability. Activity of all four samples was significantly hampered by incubation at pH

2.0, but rPatB2 was the most stable, as it retained 40% of its activity at this pH while activity of the other proteins was <10% (Fig. 5B). Activity of Solanic 200 was lower at pH 3.0–5.0 compared to the other protein

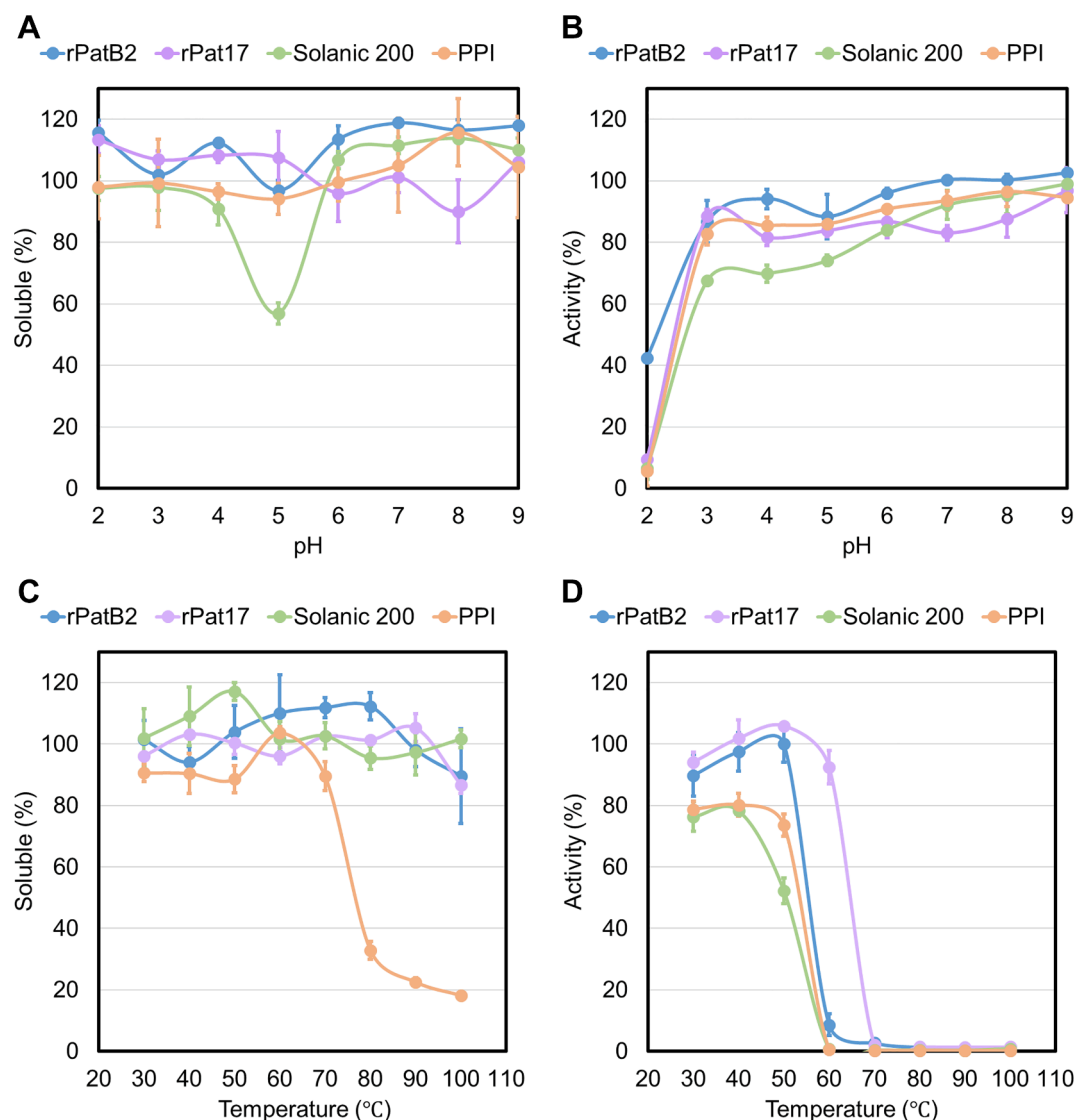


Fig. 5. Solubility and residual activity of patatins in various pH (A-B) and temperature (C-D) conditions. A and C – soluble fraction based on Bradford (mg/mL), B and D – activity based on pNP-Octanoate hydrolysis (U/mL). All values were normalized to untreated protein (either diluted in 10 mM Tris-HCl pH8.5 for pH or not heated for temperature). Measurements were performed after 30 min or 2 h incubation at various pH or temperature conditions, respectively. Blue – recombinant Patatin-B2, purple – recombinant Patatin-17, green – Solanic 200 and orange – potato protein isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples. However, the difference at these pH conditions could result from susceptibility to buffer composition rather than the specific pH directly, as these pH conditions were prepared in acetate buffer and pH 6.0–9.0 were in phosphate buffer and activities between pH 3.0–5.0 were not significantly different. High solubility and stability at acidic conditions could make rPatB2 a good candidate as protein additives for natural juices characterized by acidic pH.

Recombinant patatins and Solanic 200 exhibited excellent temperature solubility properties, >80% at 30–100 °C (Fig. 5C). PPI solubility, on the other hand, diminished at temperatures of 80 °C and above. As shown in Fig. 2B, PPI is composed of patatin and protease inhibitors. Temperature solubility of potato fruit juice, from which PPI was isolated, was previously reported to be around 80% at 20–40 °C and diminishes after incubation at 50 °C or higher (Waglay et al., 2014). Since solubility of Solanic 200, which contains patatins isolated from potato, remained soluble, it is assumed that the precipitated proteins are protease inhibitors present in the PPI sample. rPat17 was the only protein to remain fully active after 2 h at 60 °C, whereas activity of rPatB2 under these conditions was below 10% and no activity was detected for Solanic

200 or PPI (Fig. 5D). Incubation above 60 °C abolished activity of all four proteins. Both rPatB2 and rPat17 showed equal or superior solubility and stability over a wide range of pH and temperature conditions, compared to Solanic 200 and PPI.

3.6. Thermal-induced unfolding

Thermal stability was further evaluated by thermal-induced unfolding using calorimetric measurement and fluorescence differential scanning (Table 2). The “gold standard” method for thermal-induced unfolding is differential scanning calorimetry (DSC) (Vuorinen et al., 2020). As DSC measures sample energy uptake at increasing temperatures, in addition to information regarding the melting, or in the case of proteins - denaturation temperature, it also provides thermodynamic information like enthalpy, the amount of energy required to induce protein denaturation (Durowoju, Bhandal, Hu, Carpick, & Kirkitadze, 2017). Only endothermic transitions were observed in all four samples (Fig. S3), characteristic of hydrogen bond dissociation during protein denaturation (Fitzsimons, Mulvihill, & Morris, 2007). Surprisingly,

Table 2

Thermal-induced unfolding and antioxidant activity of recombinant and endo-genic patatins.

Sample	Inflection temperature measured by tryptophan fluorescence (°C)	Denaturation temperature measured by DSC (°C)	Enthalpy measured by DSC (J/g)	Antioxidant activity (mmol Trolox/g protein) ¹
rPatB2 ^a	68.3 ± 0.1 ^a	57.86 ± 0.29 ^a	2.7 ± 0.2 ^a	1.6 ± 0.2 ^a
rPat17 ^a	73.6 ± 0.2 ^b	66.59 ± 0.01 ^b	5.2 ± 0.3 ^b	1.5 ± 0.3 ^a
Solanic 200	63.8 ± 0.2 ^c	55.88 ± 0.29 ^c	2.5 ± 0.3 ^a	1.3 ± 0.2 ^b
PPI	65.7 ± 0.2 ^d	70.48 ± 0.57 ^d	2.7 ± 0.4 ^a	2.5 ± 0.3 ^c

^a Results are for two independent batches of recombinant protein.

¹ Measured by ORAC.

^a, ^b, ^c, ^d represent statistically different results within each measurement.

similar enthalpy values were obtained for Solanic 200, rPatB2 and PPI, which were isolated by different methods; these were 2-fold lower than enthalpy measured for rPat17. Schmidt et al. reported enthalpies of 1.55 J/g and 1.15 J/g for spray-dried and freeze-dried PPI, respectively; 40%

and 80% lower than Solanic 200 and PPI reported here. Solanic 200 has the lowest peak denaturation temperature, 55.9 ± 0.3 °C, of the four protein samples. Similar values of 55–57 °C were recently reported for Solanic 200 (Andlinger, Bornkeßel, et al., 2021). Peak denaturation temperature of rPat17 was 65.7 ± 1.0 °C supporting the higher stability observed for this protein after incubation at 60 °C compared to rPatB2 and Solanic 200 (Fig. 5D). PPI peak was at 70.5 ± 0.6 °C with shoulder at ~ 65 °C. A peak and shoulder thermogram of ammonium sulfate precipitated potato proteins was previously shown by Van Koningsveld et al. with slightly lower temperatures of 68 and 60 °C (Van Koningsveld et al., 2001). While enthalpies reported by Van Koningsveld et al. were much higher than those measured here, these differences could possibly result from different instrumentation and protein concentrations.

Patatins contain two tryptophan residues, which enable monitoring unfolding events by following changes in tryptophan (Trp) fluorescence intensity by differential scanning fluorescence (DSF). The inflection temperature of rPatB2 and rPat17 were the highest, at 68.3 °C and 73.6 °C, respectively, while the temperatures of Solanic 200 and PPI were 63.8 °C and 65.7 °C (Table 2). These temperatures are much higher than those measured by DSC. Two limitations of DSF are the number of Trp residues in the protein and their environment within its structure (Magnusson et al., 2019). Patatins have two Trp residues positioned only

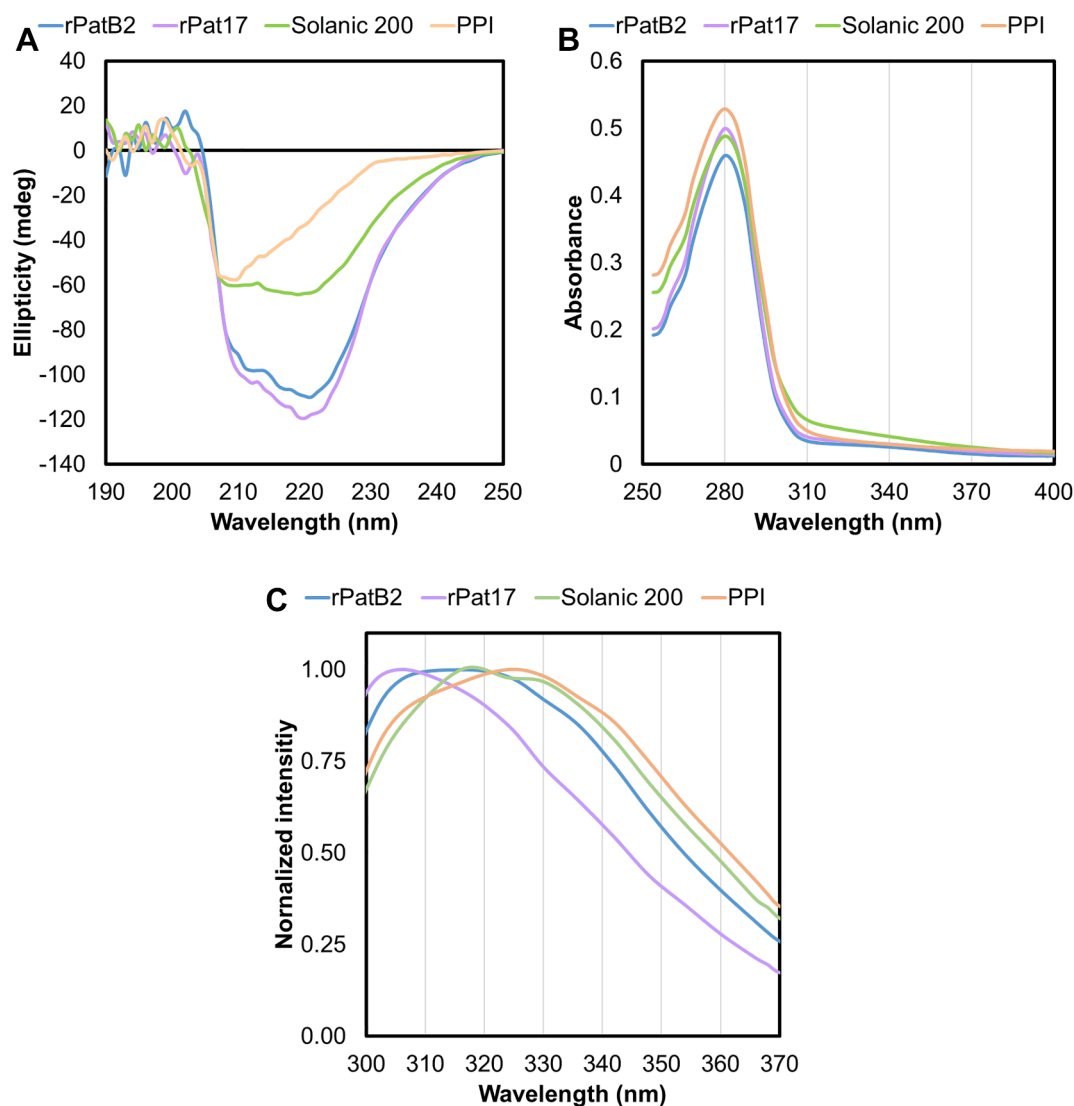


Fig. 6. Structural characterization of patatins. A. circular dichroism; B. absorbance; C. intrinsic fluorescence spectra of rPatB2 (blue), rPat17 (purple), Solanic 200 (green) and PPI (orange). Excitation and emission in C: 280 nm and 300–400 nm respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

four amino acids apart, at W255 and W260. As mature patatin contains 386 amino acids (Rosahl, Schmidt, Schell, & Willmitzer, 1986), structural changes occurring at remote areas of the protein, might not affect the fluorescence of these residues, resulting in the observed differences between denaturation temperature measured by DSC and inflection temperatures measured by DSF.

3.7. Structure

The X-ray structure of patatin-17 was solved by Rydel et al. (Rydel et al., 2003). Structures of patatins expressed in potatoes and yeast were characterized by circular dichroism (CD) spectra and intrinsic fluorescence (Fig. 6). CD spectra were used to characterize the secondary structures of the different protein samples. rPatB2, rPat17 and Solanic 200 exhibited negative peaks at 210 and 220 nm (Fig. 6A), as previously reported for isoforms A, B, and D of the patatin family (Pots et al., 1999). The lower signal obtained from the Solanic 200 can be attributed to its lower solubility compared to the recombinant proteins, as insoluble particles were removed by centrifugation before analysis. Meanwhile, the spectra of PPI was quite different than the other samples. As shown in SDS-PAGE, unlike the other sample, PPI contains a significant amount of protease inhibitors (bands of 20–25 kDa in Fig. 2B). Pouvreau et al. showed that the CD spectra of potato protease inhibitors at 20 °C starts from a minimum just under 200 nm, crosses the x-axis around 215 nm and has a positive peak at approximately 225 nm (Pouvreau, Gruppen, Van Koningsveld, Van Den Broek, & Voragen, 2005). Therefore, as the CD spectra represents an average of all the proteins in the solution, the difference observed in the spectra of PPI can be attributed to the presence of protease inhibitors in this sample. Similar helix and strand content were identified in the CD spectra of both recombinant patatins as well as the commercial protein (Table S1).

The four protein samples have similar absorbance spectra with a peak around 280 nm (Fig. 6B). Intrinsic fluorescence of a protein is affected by phenylalanine, tyrosine and tryptophan residues and is influenced by the residues environment. Exposure of these amino acids to a polar environment, causes a red-shift in their emission spectrum (Andlinger, Röscheisen, Hengst, & Kulozik, 2021). The emission spectra of rPat17 had a maxima at the shortest wavelength, suggesting it is the most tightly folded of the samples. The maximal wavelengths of Solanic 200 and PPI were at the longest wavelength. Andlinger et al. reported a shift in emission maxima between native and heat-induced aggregated patatin from 330 nm to 340 nm. The maximal intensity of PPI is at approximately 325 nm but a small shoulder can be seen in the curve just above 340 nm, suggesting the proteins in this sample may be slightly aggregated (orange curve in Fig. 6C).

Figure S4 presents visualization of the hydrophobic surfaces of Patatin-17 and Patatin-B2. As the two isoforms share 87% identity in their amino acids sequence, high similarity was observed in their hydrophobic surface. Patatin-B2 had a few very small areas in which its surface was slightly less hydrophobic than Patatin-17 (green arrows in Fig. S4B and D point at examples of such areas). Hydrophobic interactions have been shown to affect aggregation and gelation of patatins (Andlinger, Röscheisen, et al., 2021). The slight differences observed between the two recombinant proteins may affect how the proteins aggregate and gel. Therefore, hydrophobic surface information may be utilized in the future as one of the characteristics considered to select an optimal patatin isoform for a desired downstream applications.

3.8. Antioxidant capacity

PPI hydrolysate has been shown to possess excellent antioxidant capacity (Waglay et al., 2014). This capacity has been attributed, in part, to the patatin protein family (Liu, Han, Lee, Hsu, & Hou, 2003). Antioxidant capacities measured by ORAC are presented in Table 2. All four samples possessed antioxidant capacity. Capacity of the recombinant proteins was 15–20% higher than the capacity of Solanic 200, 1.5–1.6

mmol Trolox/g protein vs 1.3 mmol Trolox/g protein. PPI possessed the highest capacity, 2.5 ± 0.3 mmol Trolox/g protein, of the four samples, with similar values recently reported for patatin isolated from *S. tuberosum* (Elahi & Mu, 2017). PPI is the only protein sample which did not undergo chromatography purification, therefore, the higher antioxidant activity observed might be attributed to other potato components present in the sample. PPI was previously shown to contain ~2% polyphenols known to possess antioxidant activity. Furthermore, PPI contains a high concentration of protease inhibitors which were shown to have antioxidant capacity and are significantly lower in Solanic 200 and absent in rPatB2 and rPat17 (Liu et al., 2020). Therefore, the higher ORAC value measured in the PPI sample might be attributed to these components (Glusac et al., 2017; Wegener et al., 2015). Amino acids containing aromatic rings or sulfhydryl groups were reportedly involved in antioxidant capacity and thought to act by donating a hydrogen (Elias, Julian, & Decker, 2005). Both recombinant proteins contain 2 tryptophan and 15 phenylalanine residues. rPat17 contains 11 methionine and 17 tyrosine residues while rPatB2 contains 10 and 16, respectively. On the other hand, rPatB2 contains a cysteine residue which is not present in rPat17 (Fig. S2). The relative accessibility of these amino acids affects their ability to donate the hydrogen and can account for the small differences observed between the two recombinant proteins.

4. Conclusions

Over recent years there has been a growing interest in patatin as an alternative protein food ingredient, due to its high essential amino acids content, excellent functional properties, antioxidant capacity and lipid hydrolase activity, and most recently, its potential anti-obesity effects. Many studies have focused on optimizing the isolation and purification methods utilized to extract patatins from potato tuber. Of special interest have been issues regarding maintaining protein functionality and reducing antinutrient content while preserving high yields. In our research, we endeavored to use an alternative approach for obtaining fully functional patatins in their native form without any antinutrients which cause concern in potato isolates. The approach we chose was to recombinantly express specific patatin isoforms in *P. pastoris* with a C-terminal hexa-Histidine tag. The recombinant proteins were secreted by the yeast cells to the media from which the proteins were purified by a single affinity purification step. This approach has the potential to essentially uncouple the growing demand for patatin from potato agriculture. The protein obtained by this approach had superior purity and surface charge compared to both patatins isolated from potatoes. Each isoenzyme had unique properties as far as specific activity, substrate specificity and thermal stability, some better than those of patatin isolated from potatoes, while the antioxidant activity was comparable with commercial patatin. Not only is expression in *P. pastoris* faster than growing potatoes, but the obtained protein is reproducible and pure. This could enable the incorporation of unique traits to foods, as a specific isoenzyme can be selected for the desired trait. As patatin isolated from potato has exceptional foaming and gelling properties, further characterization of these properties is needed for the recombinant proteins. Gelation properties of rPatB2 compared to those of commercial patatins are currently under evaluation. Additionally, the flavor of recombinant patatin is still unknown and should be evaluated. Finally, this work compared only two recombinant isoenzymes. As potato tubers contain several more members of the patatin family, other isoenzymes of this family can be recombinantly expressed and characterized, some might have new or even better properties than those shown here.

CRediT authorship contribution statement

Sivan Gelley: Conceptualization, data curation, investigation, formal analysis, methodology, validation, writing-original draft, writing-review and editing. **Hodaya Lankry:** Formal analysis,

investigation, methodology. **Jovana Glusac:** Conceptualization, methodology, supervision, validation, writing-review and editing. **Ayelet Fishman:** Conceptualization, funding acquisition, resources, validation, writing-review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.130984>.

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Supplementary Information

Yeast-derived potato patatins: Biochemical and biophysical characterization

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Table S1. Secondary structure parameters determined by CD spectra

Sample	α-helix	β-strand	β-turn	Random
rPatB2[*]	30	19	17	36
rPat17[*]	33	17	17	34
Solanic 200	34	17	17	33
PPI	29	20	18	34

^{*} Average and standard deviation are for two independent batches of recombinant protein.

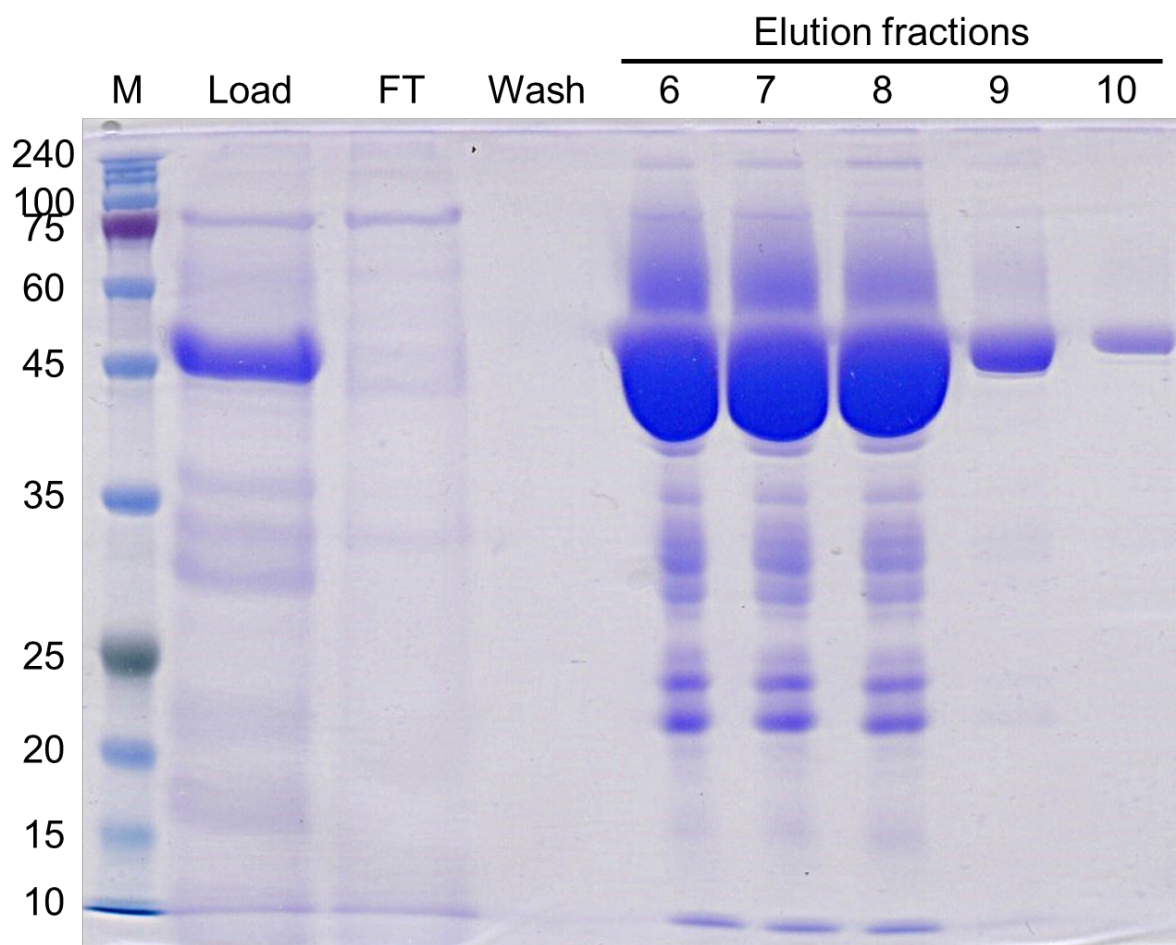


Figure S1. Typical SDS-PAGE of rPatB2 purification samples. Load – media after pH adjustment, FT – flow-through, numbers indicate different fractions in the elution step. Gel: 12% acrylamide, Load: Load, FT, wash and fractions 9-10 – 30 μ L, fractions 6-8 – 25 μ g by absorbance at 280nm.

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Patatin-B2  KLEEMVTVLSIDGGGIKGIIPAIILEFLEGQLQEVNNDARLADYFDVIGGTSTGGLLT  60
Patatin-17  QLGEVMTVLSIDGGGIRGIIPATILEFLEGQLQEMDNNADARLADYFDVIGGTSTGGLLT  60
          :* *****:***** *****:*** *****
Patatin-B2  AMITTPNENNRPFAAAKDIVPFYFEHGPHIFNYSGSILGPMYDGKYLQVLQEKLGETRV  120
Patatin-17  AMISTPNENNRPFAAAKEIVPFYFEHGQIFNPSGQILGPKYDGKYLQVLQEKLGETRV  120
          ***:*****:*****:*** **.* **** *****:*****
Patatin-B2  HQALTEVAISSFDIKTNKPVIFTKSNLAKSPELDAKMYDICYSTAAAPIYFPPHHFVTHT  180
Patatin-17  HQALTEVVISSFDIKTNKPVIFTKSNLANSPELDAKMYDISYSTAAAPTYFPPHYFVTNT  180
          *****.******:*****.****** *****:***:*
Patatin-B2  SNGARYEFNLVDGAVATVGDPALLSLSVATRLAQEDPAFSSIKSLDYKQMLLLSLGTGTN  240
Patatin-17  SNGDEYEFNLVDGAVATVADPALLSISVATRLAQKDPAFASIRSLNYKKMLLLSLGTGTT  240
          ***.******.******:*****:****:***:***:***:*****.
Patatin-B2  SEFDKTYTAEEAAKWGPLRWMLAIQQMTNAASSYMTDYYISTVFQARHSQNNYLRVQENA  300
Patatin-17  SEFDKTYTAKEAATWTAVHWMLVIQKMTDAASSYMTDYYLSTAFQALDSKNNYLRVQENA  300
          *****:***.*::***.**:***:*****:***.***.*:*****
Patatin-B2  LNGTTTEMDDASEANMELLVQVGETLLKPKVSKDSPETYEEALKRFAKLLSDRKKLRANK  360
Patatin-17  LTGTTTEMDDASEANMELLVQVGENLLKPKVSEDNPETYEEALKRFAKLLSDRKKLRANK  360
          *.*****.******:*.*****
Patatin-B2  ASH 363
Patatin-17  ASY 363
          **:

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Figure S2. Amino acid sequence alignment of mature *Solanum tuberosum* Patatin-B2 and *Solanum cardiophyllum* Patatin-17. Sequences were aligned using: <https://www.ebi.ac.uk/Tools/msa/clustalo/> Amino acid color legend: green – polar side chain, red – hydrophobic or aromatic side chain, blue – acidic side chain, magenta – basic side chain. Symbols: asterisk represent fully conserved residue, colon represents conservation with strongly similar properties and a period represents conservation with weakly similar properties. Underlined Asn residues mark predicted glycosylation sites and in bold are the catalytic Ser54 and Asp192 dyad identified in Patatin-B2.

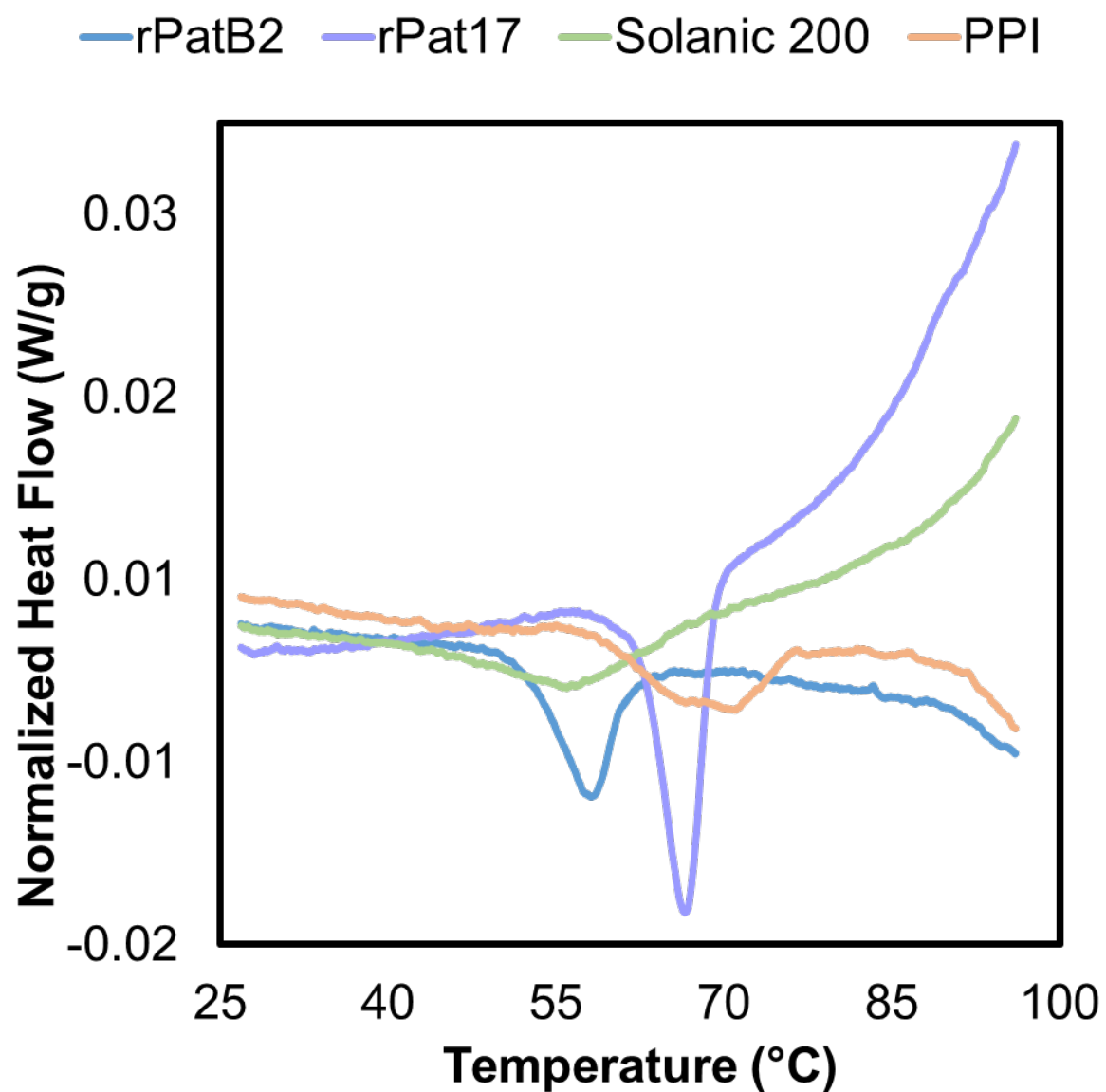
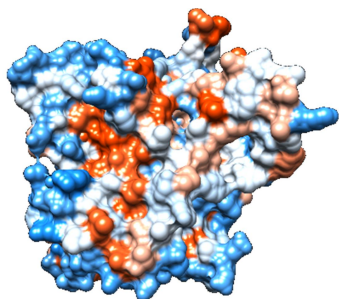
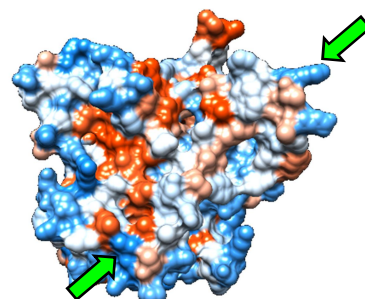


Figure S3. Differential scanning calorimetry thermograms. Recombinant Patatin-B2 (blue), recombinant Patatin-17 (purple), Solanic 200 (green) and potato protein isolate (orange).

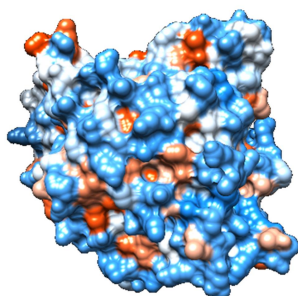
A. Pat-17



B. Pat-B2



C. Pat-17



D. Pat-B2

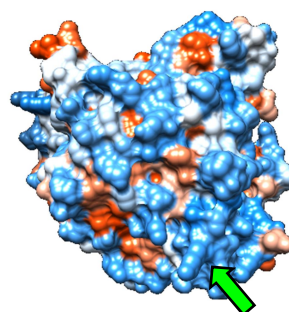


Figure S4. Hydrophobic surface of Patatin-17 (A & C) and Patatin-B2 (B & D). Patatin-17 – PDB 4PK9, Patatin-B2 – model prepared in Swiss Model server. Hydrophobic surface was visualized in Chimera 1.14 and hydrophobicity scale from red to blue - red is hydrophobic and blue is hydrophilic. A-B and C-D are the front and back views of the structures, respectively. Green arrows point at examples of less hydrophobic areas in Patatin-B2 compared to Patatin-17.