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Molecular Properties and Activities of Tuber Proteins from Starch Potato Cv. Kuras

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Potato starch production leaves behind a huge amount of juice. This juice is rich in protein, which might be exploited for food, biotechnological, and pharmaceutical applications. In northern Europe cv. Kuras is dominant for industrial starch production, and juice protein of freshly harvested mature tubers was fractionated by Superdex 200 gel filtration. The fractions were subjected to selected activity assays (patatin, peroxidase, glyoxalases I and II, α-mannosidase, inhibition of trypsin, *Fusarium* protease, and alcalase) and protein subunit size determination by SDS-PAGE and mass spectrometry. Proteins present in SDS-PAGE bands were identified by tryptic peptide mass fingerprinting. Protein complexes such as ribosomes and proteasomes eluted with the void volume of the gel filtration. Large proteins were enzymes of starch synthesis dominated by starch phosphorylase L-1 (ca. 4% of total protein). Five identified dimeric patatin variants (25%) coeluted with four monomeric lipoxygenase variants (10%) at 97 kDa. Protease inhibitor I variants (4%) at 46 kDa (hexamer) inhibited alcalase. Fourteen Kunitz protease inhibitor variants (30%) at 19 kDa inhibited trypsin and *Fusarium* protease. Carboxypeptidase inhibitor variants (5%) and defensins (5%) coeluted with phenolics. The native sizes and molecular properties were determined for 43 different potato tuber proteins, several for the first time.

KEYWORDS: ***Solanum tuberosum*; potato tuber proteins; patatin; protease inhibitor; lipoxygenase; glyoxalase; mannosidase**

INTRODUCTION

Potato (*Solanum tuberosum*) is the world's fourth most important crop after rice, wheat, and corn, and its importance is growing (*1*). The potato tuber is also an important source of industrial starch. In northern Europe, the Kuras cultivar is the major starch potato due to a high yield and superior pest resistance. Potato juice is a waste product from industrial starch manufacture and contains approximately 1.5% (w/v) of soluble protein. Potato juice protein has been classified into three major groups: patatins, protease inhibitors, and other proteins (*2*). Patatins and protease inhibitors, each comprising approximately 40% of total tuber protein, are mostly considered as storage proteins, in addition to their lipolytic and inhibitory activities, respectively. Potato juice is a potential resource of huge quantities of novel proteins for specific biotechnological, pharmaceutical, or food applications, in addition to the present application as feed supplement. Realizing this potential requires in-depth knowledge of the molecular structures, activities, and quantities of the individual proteins present and development of efficient but gentle separation technology.

Patatins and protease inhibitors have been fractionated and characterized by biochemical and biophysical methods, whereas only scattered knowledge is available for the other major proteins. Patatin is a group of similar storage glycoprotein variants with very broad lipid acyl esterase activity. Pots et al. (*3*) separated the patatins of cv. Bintje into four pools according to chromatographic and electrophoretic characteristics. The biophysical properties of the pools were very similar. In contrast, the potato tuber protease inhibitors constitute a very diverse group of proteins, inhibiting a variety of proteases (*4*, *5*) and other enzymes (*6*). They differ in their amino acid sequences, chain lengths (M_r 4, 8, 13, and 20−22 kDa), and subunit compositions (monomer to hexamer) (*2*, *4*, *5*, *7*, *8*). Cv. Kuras tuber contains five nonhomologous families of protease or peptidase inhibitors (H. V. Nielsen and K. G. Welinder, unpublished results), I3A Kunitz peptidase inhibitor (KPI), I13 peptidase inhibitor I (PI I), I20 peptidase inhibitor II (PI II), I25 multicystatin peptidase inhibitor, and I37 carboxypeptidase inhibitor (CPI), according to the MEROPS classification (*9*, *10*). Erroneous assignment of potato inhibitor class is common in the literature, as is homology-based automated assignment of

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specific peptidase inhibitory activity in high-throughput transcriptome and proteome analyses. A further challenge to the exploitation of potato juice protein from starch production is a pronounced variability in protein abundance (*11*) and variability of amino acid sequence of patatins (*8*) and Kunitz protease inhibitors (*12*, *8*) among potato varieties. Specific inhibitory activities were only recently linked directly to unique amino acid sequences encoded by seven heterologously expressed KPIs (*5*) and one PI I (*7*).

Hundreds of the group of other proteins present in potato tuber have lately been characterized and identified in their denatured form in proteome studies. An extensive comparative two-dimensional gel electrophoresis-based proteome study was done on eight genetically modified (GM) lines, and 32 non-GM potato genotypes, including 21 named cultivars of tetraploid potato, 8 landraces, and 3 diploid genotypes (*11*). A study of the major proteins present in cv. Kuras also used two-dimensional (2D) gel electrophoresis (*8*) and demonstrated that the amino acid sequences of the predominant groups of patatin and Kunitz protease inhibitors were cultivar dependent. In the same cultivar the protein profiles of amyloplast and starch granule (*13*) and vacuole have been performed (M. Jørgensen and K. G. Welinder, unpublished results).

The aim of the present work was to characterize, identify, and quantify all major proteins present in cv. Kuras potato juice in their native functional form, using gentle gel filtration fractionation and selected activity assays (patatin, peroxidase, glyoxalase I and II, α-mannosidase, and protease inhibition). Gel filtration also recovers the very large and small proteins lost in 2D gel electrophoresis. The functional proteins and protein complexes were analyzed by direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and nonreducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), which both determined the M_r of covalently linked subunits. The SDS-PAGE-separated protein bands were identified by tryptic peptide mass fingerprinting (PMF).

MATERIALS AND METHODS

Plant Material. Field-grown starch potatoes (*Solanum tuberosum* cv. Kuras) were obtained from AKV-Langholt Amba (Vodskov, Denmark). The tubers were freshly harvested in October after natural senescence.

Preparation of Potato Tuber Juice. Potato tubers were minced at 4 °C in a domestic juice extractor in the presence of sodium bisulfate (1 g/kg of tuber) to prevent browning. Starch sedimented for 30 min at 4 °C, and the supernatant was decanted and centrifuged at 10000*g* for 30 min at 4 °C. The juice was filtered through a 0.22 μm filter and concentrated approximately 10-fold by dry Sephadex G25 (1 g/4 mL of juice). Materials and equipment are from Amersham Biosciences, Uppsala, Sweden, except if otherwise mentioned. The Sephadex absorbed all visible liquid in 30 min at 4 °C. Centrifugation of the gel at 10000*g* for 15 min at 4 °C and filtration (0.22 μm) gave a clear protein sample of 14.3 mg/mL using bovine serum albumin as protein concentration reference (*14*).

Tuber protein was fractionated in 50 mM sodium phosphate buffer, 40 mM NaCl, pH 7.0, at room temperature by gel filtration on a Superdex 200 HR 10/30 column using an Äkta Purifier chromatograph. Effluent was monitored by absorbance at 280 and 310 nm and by conductivity measurement. The column was calibrated with blue dextran (void volume), thyroglobin (669 kDa), ferritin (440 kDa), catalase (232 kDa), ovalbumin (43 kDa), and horse skeletal myoglobin (17.5 kDa). Ovalbumin and myoglobin were from Sigma-Aldrich (St. Louis, MO).

Protein and Enzyme Assays. All analyses were carried out in triplicate. Protein concentration of gel filtration fractions was determined by the bicinchoninic acid assay (BCA) using bovine serum albumin as standard (*15*) and by absorbance ($A_{280nm} - A_{310nm}$) assuming 1 mg of protein/mL gives $A = 1.0$.

Protease Inhibition. Three proteases were used: alcalase, porcine trypsin, and *Fusarium* protease (gifts from Novozymes, Denmark). One volume of inhibitor sample and 3 volumes of protease in 0.01% (v/v) Triton X-100 were preincubated for 15 min at 37 °C. A crushed tablet of blue casein (Protazyme AK substrate, Megazyme, Dublin, Ireland) in 3 mL of 0.01% (v/v) Triton X-100 was used as protease substrate. One hundred microliters of this substrate and 100 μL of assay buffer (100 mM Tris, pH 9.0) were added per well of a cold (0 °C) 96-well filter plate (0.45 μm; Millipore, Bedford, MA). Proteolysis was initiated in the wells by adding 4 μL of the preincubated protease−inhibitor mixture. After 15 min at 37 °C with strong shaking (at least 240 rpm), the plate was centrifuged immediately at 0 °C and 800*g* for 4 min, and the blue filtrates were collected into a microtiter plate (I. Idel and K. G. Welinder, unpublished results). The absorbance was measured at 600 nm. Protease activity was assayed by substituting inhibitor sample by 0.01% (v/v) Triton X-100 for reference, and protease concentrations were adjusted to give an absorbance of 1 at 600 nm. Inhibitory activity was scored as "+" at 35% inhibition or better at the indicated assay conditions.

α-Mannosidase. α-Mannosidase (EC 3.2.1.24) was assayed according to the method of ref *16*. A stock solution of 100 mM *p*-nitrophenyl mannoside substrate in *N*′,*N*′-dimethylformamide (both Sigma-Aldrich) was stored at −20 °C. Sample, 50 μL, was mixed with 150 μL of 5 mM *p*-nitrophenyl mannoside in 50 mM sodium citrate, pH 4.5, and incubated for 45 min at 37 °C. The reaction was terminated by 75 μL of 1 M sodium carbonate buffer, pH >10, and the absorbance measured at 405 nm. α-Mannosidase activity in units was calculated using $\epsilon_{405} = 18.5\ \mu M^{-1}\ cm^{-1}$ for released *p*-nitrophenol. One unit gives 1 pmol of product/h.

Lipid Acyl Hydrolase (Patatin). The patatin assay was also based on released *p*-nitrophenol (*16*). Samples were diluted in 30 mM Tris-HCl, pH 8.2. A stock solution of 100 mM *p*-nitrophenyl laurate substrate in *N*′,*N*′-dimethylformamide (Sigma-Aldrich) was stored at −20 °C. One volume of sample was equilibrated at 30 °C before 1 volume of 11 μM *p*-nitrophenyl laurate in the same buffer was added. After incubation for 5 min at 30 °C, the reaction was stopped by the addition of 1 volume of 0.8 M sodium carbonate buffer, pH >10, and the absorbance measured at 405 nm. Lipid acyl hydrolase activity was calculated as for α-mannosidase, except that 1 unit gives 1 nmol/h.

Peroxidase. Protein sample was mixed with 20 mg/mL *o*-phenylenediamine, 0.006% (v/v) H_2O_2 in 25 mM sodium citrate at pH 5.0 at 1:1 v/v. After incubation for 1 min at room temperature, the reaction was stopped by the addition of 1 volume of 1 M H_2SO_4. Activity was measured as the increase in absorbance at 450 nm/min (*17*), and 1.0 absorbance unit was defined as 1 unit of enzyme.

Glyoxalases I and II. Glyoxalase I was assayed as described in ref *18*. The substrate hemithioacetal was formed by stirring 7 mM methylglyoxal and 6.7 mM GSH (Sigma-Aldrich) in 0.1 M Mes−NaOH buffer, pH 6.5, for 30 min at 30 °C. The initial concentrations of methylglyoxal and GSH were chosen to give a final substrate concentration of 2 mM in the assay mixture of 400 μL, assuming a dissociation constant of 3.0 mM for hemithioacetal (*18*). Protein sample was diluted with the above buffer 3:1 and added to the preformed substrate. The formation of *S*-D-lactoylglutathione at 30 °C was measured at 240 nm after 10 min and calculated from $\Delta\epsilon_{240} = 2.86\ mM^{-1}\ cm^{-1}$ (*18*). One unit of enzyme gives 1 mmol/h of product.

Glyoxalase II was assayed in the same buffer as glyoxalase I (*18*). Protein sample, 50 μL, was preincubated with 20 μL of 7.5 mM 5′,5′-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich) in 0.1 M buffer for 15 min at room temperature, and the reaction was started by the addition of 10 μL of 37.5 mM *S*-D-lactoylglutathione, giving a final assay concentration of 3 mM, at which concentration a linear response was observed after ca. 10 s. Formation of 2-nitro-5-thiobenzoic acid was followed at 405 nm for 20 min; $\Delta\epsilon_{412} = 13.6\ mM^{-1}\ cm^{-1}$ was used to calculate product concentration (*18*). One unit of glyoxalase II activity gives 1 mmol/h of 2-nitro-5-thiobenzoic acid.

SDS-PAGE. Samples for SDS-PAGE corresponding to 25 μg of protein (BCA assay) were boiled in nonreducing sample buffer for 3 min. The stacking gel was 3%, and separation gels were 7.5 or 18%

(*19*). Gels were stained with epicocconone (*20*) alias Deep Purple, and the fluorescent protein bands were visualized in a Typhoon 8600 scanner and ImageQuant version 5.1 (Molecular Dynamics, Sunnyvale, CA).

In-Gel Trypsin Digestion. In-gel digestion was performed essentially as described in refs *8* and *21*. SDS-PAGE-separated protein bands were excised, chopped in small cubes with a razor blade, washed extensively in 0.1 M NH_4HCO_3 and 0.1 M NH_4HCO_3/acetonitrile (1:1), and dehydrated in acetonitrile. The dehydrated gel pieces were reswollen in 25 μL of 12.5 ng/μL of sequencing-grade modified porcine trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3, pH 8.0, at 4 °C for 45 min prior to overnight digestion at 37 °C. Peptides were extracted by 10 μL of 5% formic acid for 15 min and incubated again at the same conditions prior to the addition of 10 μL of acetonitrile for another 15 min. The two extracts were pooled and concentrated to 1−2 μL in a vacuum centrifuge before the addition of 20 μL of 5% formic acid. Peptides were then analyzed by mass spectrometry or stored at −20 °C.

MALDI-TOF MS. Protein samples were prepared according to the drying droplet method (*22*) using saturated sinapinic acid (3,5-dimethethoxy-4-hydroxycinnamic acid) in 0.1% trifluoroacetic acid (TFA) in water/acetonitrile (2:1, v/v) as matrix. Equal volumes of sample and matrix were mixed, deposited on the target plate, and air-dried prior to analysis. MALDI-TOF MS analysis in the linear mode was performed using a Reflex III instrument (Bruker Daltonics, Bremen, Germany). Spectra were externally calibrated using cytochrome *c* (12360 Da, Sigma-Aldrich), carbonic anhydrase (29000, Sigma-Aldrich), and bovine serum albumin (66430 Da, Sigma-Aldrich). The mass spectra were annotated with Moverz software (Genomic Solutions, Ann Arbor, MI).

Peptides were analyzed by MALDI-TOF MS using 2,5-dihydroxybenzoic acid (DHB; Sigma-Aldrich) as matrix. The DHB was dissolved in 0.1% TFA/ acetonitrile (2:1, v/v) at 3 g/L. In-gel-digested tryptic peptides were subjected to reversed-phase purification in microcolumns prepared in GelLoader pipet tips with constricted outlets. Five microliters of Poros R2 beads (10 μm, Applied Biosystems, Foster City, CA) suspended in methanol was loaded into the microcolumn and packed by air pressure from a syringe. The column height was 1−2 mm. The resin was equilibrated by 20 μL of 5% formic acid passed through the column by air pressure. Sample (1−5 μL) was loaded and washed with 20 μL of 5% formic acid. Peptides were eluted with 1 μL of DHB matrix solution and deposited directly onto an anchor chip target plate (hydrophobic surface; Bruker Daltonics) in 4−6 small droplets of mixed sample and matrix. The targets were air-dried prior to MALDI-TOF MS.

The peptides were analyzed on the REFLEX III instrument set at delayed extraction and positive reflectron mode. Mass spectra were calibrated by the monoisotopic masses of either an external standard of angiotensin II (1046.54 Da), bombesin (1619.82 Da), ACTH peptide 18−39 (2465.20 Da), and somatostatin (3147.46 Da) (Sigma-Aldrich) or an internal standard of angiotensin II and the autocatalytic trypsin peak at 2211.11 Da. The mass spectra were annotated using Moverz software. The monoisotopic *m*/*z* values were annotated if (i) the signal of the main isotopic peak was at least 3 times higher than the signal-to-noise ratio, (ii) at least three isotopic peaks were visible above noise, and (iii) the intensity distribution of these isotopic peaks was as predicted for the specified *m*/*z* value.

Protein Identification. The VEMS software package (*23*, *24*) was used for protein identification. Protein and peptide databases were created by VEMSdata from translation of all available potato EST sequences (TIGR, release 10.0, November 2004, http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=potato), and from Kuras tuber-specific expressed sequence tag (EST) sequences (*25*) (Kuras contigs available at http://www.bio.aau.dk/en/st-data.htm). The experimental peptide mass lists of the protein digests were searched in these local protein databases using the VEMSmaldi application. The mass accuracy was set to 0.5 Da. Two missed trypsin cleavages were allowed. Moreover, the peptide mass lists were searched by Mascot 2.1 (Matrix Science, London, U.K.) using the same databases and settings, including oxidized methionine. Proteins with a VEMS or Mascot significance of $p < 0.05$ were accepted as identified. The top-ranking proteins of the databases were manually examined with GPMAW software (Lighthouse Data, Odense, Denmark) (*26*) searching for additional tryptic peptides matching the MS-measured masses.

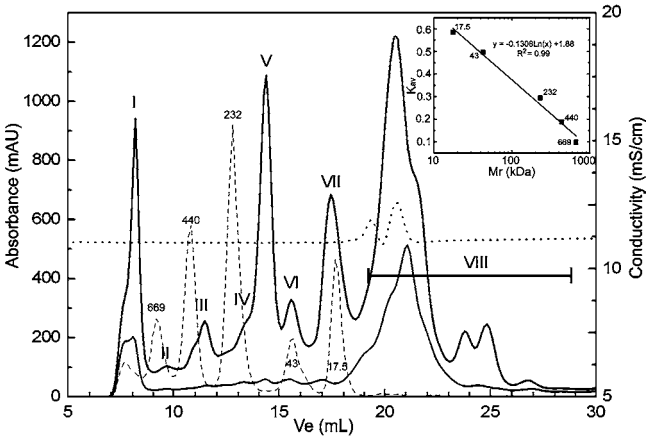

Figure 1. Superdex 200 gel filtration of potato juice proteins concentrated approximately 10-fold. Column total volume, 24.0 mL; void volume, 7.5 mL; 0 mL at initiation of sample loading; buffer, 50 mM sodium phosphate with 40 mM NaCl, pH 7.0; flow rate, 0.2 mL min^{-1}; protein sample size, 250 μL. Eight fractions were collected: fractions I (7.0–9.0 mL), II (9.0–10.5 mL), III (10.5–12.5 mL), IV (12.5–14.0 mL), V (14.0–15.0 mL), VI (15.0–16.5 mL), VII (16.5–18.0 mL), and VIII (18.5–29.0 mL). Black line, 280 nm; gray line, 310 nm; black dotted line, conductivity. Salt eluted at 20.0 mL. External calibration of the column with dextran blue and standard proteins is indicated in a thin dashed line, and the molecular masses (kDa) are shown at the corresponding peaks. (Inset) $K_{av} = V_e - V_o/V_t - V_o$ versus M_r (kDa) for the column.

RESULTS AND DISCUSSION

Experimental Design. Native proteins of potato juice from field-grown Kuras tubers at harvest were fractionated according to their sizes by Superdex 200 gel filtration and pooled into fractions I−VIII (**Figure 1**). The fractions were analyzed for protein content, selected enzymatic or inhibitory activities (**Table 1**), relative abundance by SDS-PAGE (**Figure 2**), and subunit size by SDS-PAGE and MALDI-TOF MS (**Figure 3**; fractions I−IV did not fly well in MS). Furthermore, the proteins of 62 visible epicocconone-stained protein bands cut from the nonreducing SDS-PAGE gels were assigned to 90 proteins by tryptic peptide mass fingerprinting (PMF) analysis. All peptide data were curated manually (**Table 2**; Supporting Information Table S1). Forty-three different Kuras tuber proteins were identified and characterized in this way, and their properties were related to known biological functions. Major proteins such as starch phosphorylase L-1, patatins, lipoxygenases, and Kunitz protease inhibitors were identified as background in several gel filtration fractions and SDS-PAGE gel bands. SDS-PAGE band intensity (**Figure 2**) and the number of peptides assigned to a protein (**Table 2**) show that the primary appearance of starch phosphorylase L-1 was in fraction III, patatins and lipoxygenases were together in fraction V, and Kunitz protease inhibitors were in fraction VII. The following paragraphs comment on a selection of proteins unique to each gel filtration fraction.

Fraction I. Fraction I of the gel filtration contains complexes and very large proteins passing a 0.22 μm filter, but was excluded from Superdex 200. The enzymatic activities of fraction I might reflect unspecific aggregation or functional complexes (**Table 1**). Three ribosomal proteins of approximately 30 kDa were identified in fraction I, indicating that they may be present in ribosomal particles. The PMF method will identify only well-separated abundant potato proteins of known sequence

Table 1. Molecular Masses Derived from Superdex 200 Gel Filtration (**Figure 1**) and Selected Activities of Native Tuber Proteins[a]

		fraction from gel filtration, **Figure 1**							
		I	II	III	IV	V	VI	VII	VIII
M_r, peak/midpoint of fraction (kDa)		>1700/1800	810/810	340/340	130/145	97/78	46/42	19/21	–
elution volume (mL)		7–9	9–10.5	10.5–12.5	12.5–14	14–15	15–16.5	16.5–18	18–29
protein, BCA/$A_{280-310nm}$ (mg)		1.56/0.42	0.33/0.11	0.63/0.29	0.63/0.36	1.21/0.69	0.41/0.32	1.23/0.64	(7.3/2.0)
activity (units)	lipid acyl hydrolase (patatin)	3.28 ± 0.16	0	0.23 ± 0.01	0.94 ± 0.12	5.80 ± 0.55	0.46 ± 0.12	0.54 ± 0.13	0
	peroxidase	0	0	0	0	2.8 ± 0.5	9.4 ± 0.5	23.9 ± 2.2	0
	α-mannosidase	0	0	20.7 ± 1.4	0	0	0	2.15 ± 0.37	14.7 ± 4.4
	glyoxalase I	5.9 ± 1.2	0	0	0	0.68 ± 0.12	0.69 ± 0.12	1.12 ± 0.12	0.51 ± 0.00
	glyoxalase II	68.8 ± 4.8	0	7.7 ± 0.8	3.7 ± 0.1	0.67 ± 0.01	0.52 ± 0.08	241 ± 4	35 ± 5
inhibition	alcalase/subtilisin (S8)	–	–	–	–	–	+	–	–
	Fusarium protease (S1A)	–	–	–	–	–	–	+	–
	trypsin (S1A)	–	–	–	–	–	–	+	–

[a] Protease classes indicated in parentheses (9–10). Inhibitor activity present (+) or absent (–).

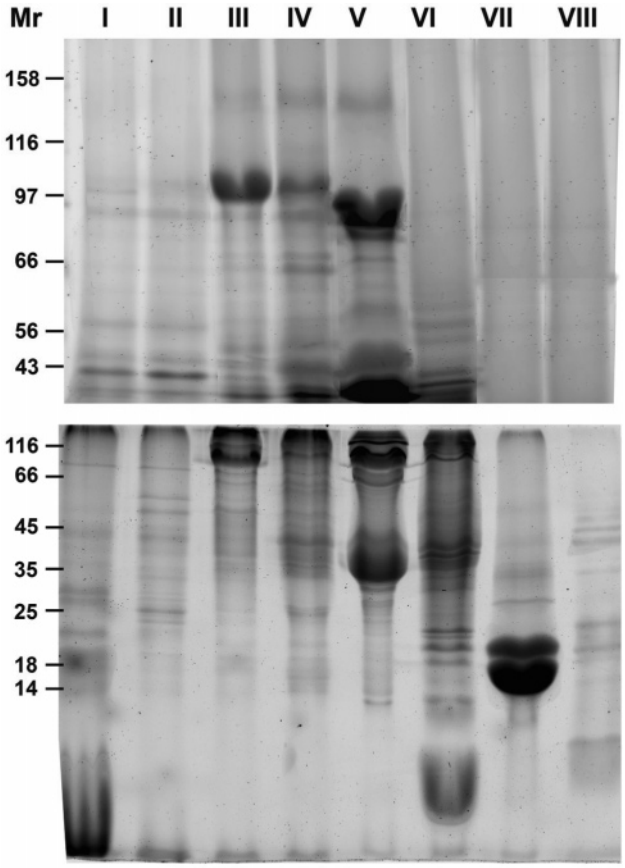

Figure 2. SDS-PAGE of gel filtration fractions I–VIII of potato juice. Marker protein sizes (kDa) are indicated to the left. A 7.5% gel (top) and an 18% gel (bottom) are shown. Fluorescent epicocconone staining (Deep Purple) was used.

and not all ribosomal subunits. Likewise, the identification of a 58 kDa proteasome ATPase subunit indicates the presence of 26S proteasome particles. Proteasomes degrade ubiquitin-labeled proteins to short polypeptides and amino acids in an ATP-driven reaction (*27*). A respiratory burst oxidase unit of a calculated M_r of 108.5 was identified at 37 kDa in fraction I and at 50 kDa in II. The reason for this discrepancy is unknown, but the data indicate that the protein was most likely present as background in these bands, yet giving a significant number of matching peptide masses. The identified respiratory burst oxidase in potato has recently been shown to be part of a NADPH-dependent O_2^--generating complex associated with the plasma membrane (*28*), which is in agreement with the large native size. The four 15−27 kDa "unknowns" of fraction I are most likely hitherto unidentified subunits of various cellular particles. Thus, their presence in fraction I provides only the first hint that they function in concert with other proteins.

Fraction II. Two proteins were found only in fraction II, the α and β subunits of plastidic chaperonin 60 (Cpn 60). This protein-folding complex is thought to consist of 14 β subunits, which can assemble in the presence of Mg-ATP (*29*). This scaffold appears to be necessary for the assembly of 14 α subunits completing the functional Cpn 60 complex. The calculated M_r of Cpn 60 is approximately 840 kDa (assuming that the M_r values of the small cpn10 subunits and the plastidic target signals are similar). This M_r fits well with the M_r of 810 kDa for fraction II proteins (**Table 1**).

Fraction III. Fraction III (and IV) gave rise to a major SDS-PAGE band at 105−110 kDa, identified as α-1,4 glucan phosphorylase L-1, alias starch phosphorylase L-1 (SP L-1). This dominant protein was also found at 140−150 kDa, apparently smearing during PAGE. In addition, SP L-1 associated with other proteins in fractions I and II (**Table 2**). SP L-1 was also the major protein from tuber starch granules and amyloplasts from Kuras (*13*). In potato, two forms of SP have been identified, SP L-1 and L-2 (*30*), where the L-1 form exists as both homodimer and heterodimer with the SP L-2. SP L-2 occurs almost exclusively as the heterodimer (*31*). In the present study SP L-2 was not identified, probably due to low abundance and dominance of the peptides originating from SP L-1. Also, in isolated amyloplasts SP L-2 was very minor compared to SP L-1 (*13*). The predominance of SP L-1 in fraction III agrees with SP L-1 being a homodimer (or trimer) of 340 kDa.

α-Glucan water dikinase (GWD, formerly R1; EC 2.7.9.4) is involved in starch biosynthesis and present in the amyloplast and starch granules (*13*). GWD was present in fractions III and IV and showed a subunit M_r of 140−150 kDa. The gel filtration elution volume of GWD confirms a homodimeric native structure of 330 kDa, as found recently (*32*).

α-Mannosidase activity was found mainly in fraction III (**Table 1**), but its presence could not be confirmed by PMF, indicating that it is a minor component of potato juice. Potato contig TC126949 (www.tigr.org) translates into an α-mannosidase subunit of 65.9 kDa, whereas gel filtration corresponded to a native mass of 340 kDa (**Table 1**). Hence, potato α-mannosidase is a multimer, possibly a tetramer or pentamer.

Two members of the heat shock family 90 were identified, Hsp90 at 70 and 75 kDa in fraction III and Hsp90-2-like at 70 kDa in fraction IV. Both have calculated M_r values of 80 kDa. The gel filtration elution volume of Hsp90-2-like indicates a

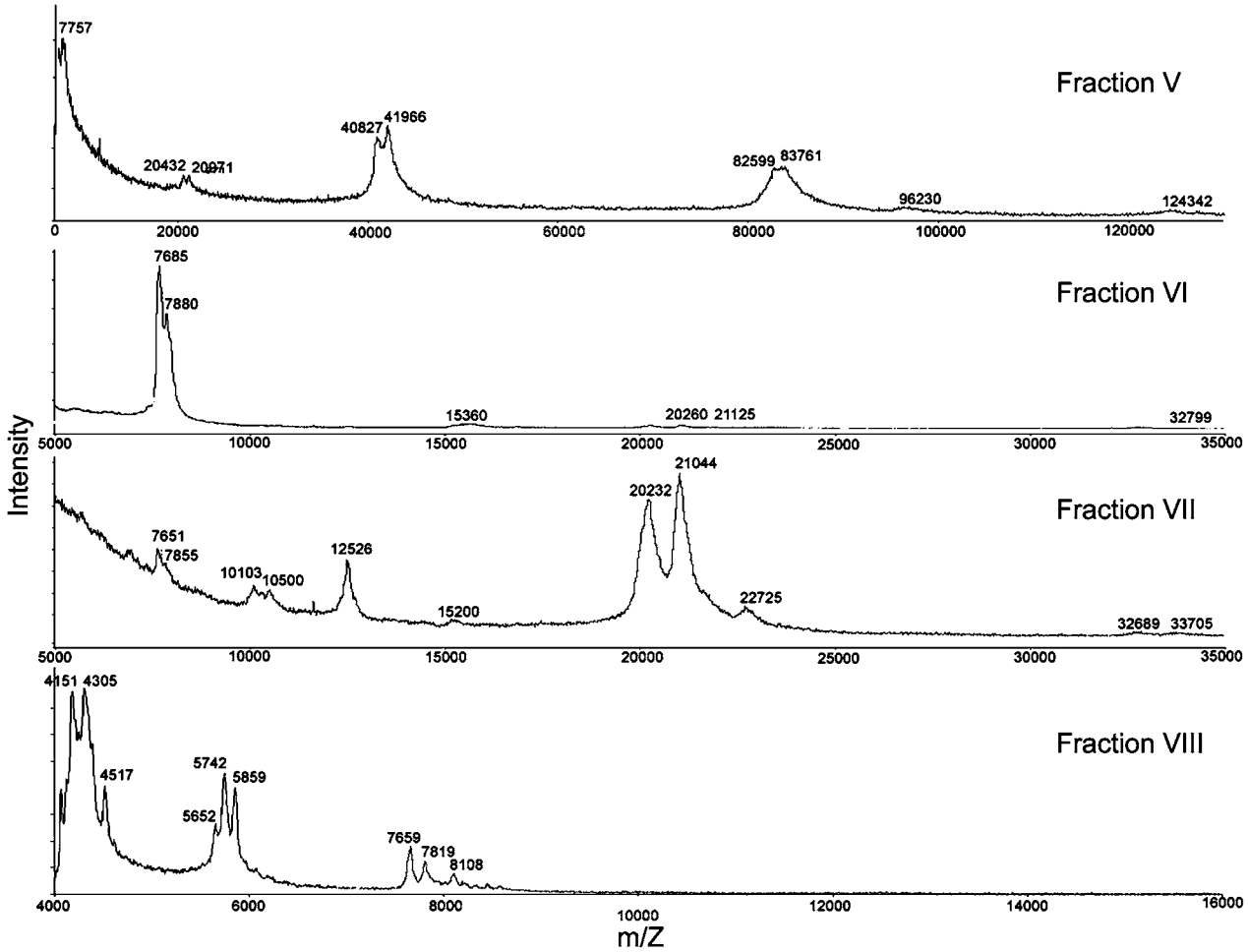

Figure 3. MALDI-TOF mass spectra of gel filtration fractions V–VIII (from **Figure 1**).

complex size of 340 kDa, whereas the Hsp90 complex might be only half this size.

Fraction IV. An SDS-PAGE band of 60 kDa was identified as 4-α-glucanotransferase (D-enzyme). D-Enzyme was first found in potato tubers in 1953 (*33*) and later characterized (*34*). However, its function in starch metabolism is still unknown. In gel filtration the D-enzyme eluted with a native mass of 130−145 kDa, showing for the first time that potato D-enzyme is most likely a dimer.

Fraction V. Fraction IV has a significant overlap with fraction V, which is dominated by lipoxygenases (100 kDa), and patatins (40 kDa) (**Figure 2**). Acyl lipid hydrolase activity confirmed the presence of patatin, which retained full activity during gel filtration (**Table 1**). The elution volume confirmed that active patatin is a dimer of approximately 97 kDa (*3*). MALDI-TOF MS (**Figure 3**) showed a broad and split mass peak of single charged patatin subunits at 41−42 kDa in agreement with the five patatin variants identified in fraction V (**Table 2**). Doubly charged subunits (21 kDa) and singly charged dimers (83 kDa) are commonly seen in this type of MS.

The superfamily of lipoxygenases (LOX) constituted the second major group of proteins in fraction V as seen by SDS-PAGE. LOX eluted mainly as monomers and fits a weak signal in MALDI-TOF MS at 96 kDa. Four variants were identified in fraction V, and a fifth was identified in fraction VI. The major LOX variant (TC112595) was also identified in 75 and 68 kDa SDS-PAGE bands, presumable in N-terminally truncated forms as indicated by the peptide coverage (Supporting Information Table S1). The LOX nomenclature of Lutteke et al. (*35*) is used. LOX is recognized by an iron-binding motif, His-X_4-His-X_4-His-X_{17}-His-X_8-His, and catalyzes the dioxygenation of fatty acids. The high accumulation of LOX in Kuras (approximately 10% of the total protein) suggests that plant lipoxygenases might also serve in the temporary storage of nitrogen during vegetative growth, as suggested for soybean LOX (*36*).

Fraction VI. The peak of gel filtration fraction VI eluted as the ovalbumin standard at 43 kDa (**Figure 1**) and inhibited alcalase (subtilisin) strongly (**Table 1**). The major SDS-PAGE band had a mass of 7.6−7.9 as shown by MS (**Figure 3**), and two variants of PI I (protease inhibitor I) were identified by PMF analysis (**Table 2**). Therefore, active Kuras tuber PI I appears to be a pentamer or hexamer as previously found in potato (*7*, *37*). We have recently identified seven different PI I variants among soluble vacuolar proteins isolated from Kuras (M. Jørgensen and K. G. Welinder, unpublished data). In cv. Elkana (*4*) eight isoforms of PI I, characterized by their isoelectric points between pH 5.1 and 7.8, showed some inhibitory activity for trypsin (40−80 mg of trypsin/g of inhibitor) and less for chymotrypsin (10 mg of chymotrypsin/g of inhibitor). Subtilisin was not tested in that study and might also be the better target protease of Elkana PI I variants.

Peroxidase activity was found in fractions VI and VII (**Table 1**). However, peroxidase was a minor component in tuber not identified by the molecular methods. A peroxidase was previously identified as TC111720 (*8*).

Glyoxalases I and II. The activity of glyoxalase I was found mainly in fractions I (aggregate or complex) and VI, indicating

Table 2. Identification of Potato Tuber Proteins Fractionated by Superdex 200 (**Figure 1**, Fractions I–VIII) and SDS-PAGE (**Figure 2**)[a]

accession no.	protein function	peptides (no.) VEMS/ Mascot	score VEMS/ Mascot	signifi-cance VEMS	M_r SDS-PAGE (kDa)	M_r calcd (kDa)	coverage (%) VEMS/ Mascot
		Fraction I					
TC119041	α-1,4 glucan phosphorylase L-1 (EC 2.1.1.4)	19/19	40/157	0.000	105	104.4	30/30
TC127939	unknown	7/7	13/74	0.000	62	62.4	16/16
TC126136	26S proteasome regulatory particle triple A ATPase subunit 2	6/6	13/45	0.044	58	49.5	15/15
AB064343	respiratory burst oxidase protein F	11/11	24/80	0.000	37	108.5	17/17
TC125898	40S ribosomal protein S2	8/11	17/111	0.024	30	30.4	35/41
TC116056	unknown	4/10	8/133	0.002	29	27.8	17/38
TC119161	60S ribosomal L13	8/10	17/92	0.000	27	23.6	39/49
CV469081	unknown	5/10	10/95	0.250	27	28.4	24/44
CN215334	unknown	7/8	15/117	0.000	16	24.0	46/47
TC131597	unknown	9/8	10/102	0.000	15	30.1	45/40
		Fraction II					
TC119041	α-1,4 glucan phosphorylase L-1	17/17	35/24	0.000	100	104.4	25/25
TC112107	9–13 lipoxygenase (EC 1.13.11.12)	31/31	64/183	0.000	100	96.9	44/43
TC112595	lipoxygenase (EC 1.13.11.12)	27/26	58/141	0.000	100	96.8	37/37
TC112798	9-lipoxygenase (EC 1.13.11.12)	27/26	51/123	0.000	100	97.3	39/39
TC112465	lipoxygenase-2 (EC 1.13.11.12)	26/25	54/122	0.000	100	96.9	32/32
TC112595	lipoxygenase	13/14	28/130	0.000	95	96.8	20/21
TC112135	Cpn60 α subunit	15/17	32/199	0.000	65	55.5	41/40
TC119088	Cpn60 β subunit	12/16	26/173	0.005	60	57.7	28/37
AB064343	respiratory burst oxidase protein F	12/12	26/107	0.001	50	108.5	18/18
TC120533	unknown	5/5	11/75	0.017	30	29.2	33/33
		Fraction III					
TC119041	α-1,4 glucan phosphorylase L-1	31/31	65/145	0.000	150	104.4	41/38
TC126433	α-glucan water dikinase (GWD, EC 2.7.9.4)	27/27	59/98	0.000	150	155.6	22/22
TC119041	α-1,4 glucan phosphorylase L-1	28/29	55/148	0.000	140	104.4	36/34
TC126433	α-glucan water dikinase GWD	22/25	47/72	0.000	140	155.6	20/22
TC112041	α-1,4 glucan phosphorylase L-1	37/38	77/308	0.000	110	104.4	48/49
TC112107	9–13 lipoxygenase	30/32	60/183	0.000	100	96.9	42/43
TC112595	lipoxygenase	25/28	53/141	0.000	100	96.8	31/35
TC119041	α-1,4 glucan phosphorylase L-1	19/19	35/40	0.001	100	104.4	23/24
TC118998	Hsp90-2-like	17/17	34/129	0.000	75	80.3	19/19
TC118998	Hsp90-2-like	13/13	26/100	0.005	70	80.3	18/18
DQ114417	patatin, pat2-k1	7/7	14/83	0.009	45	40.0	29/29
		Fraction IV					
TC126433	α-glucan water dikinase GWD	25/25	51/184	0.000	150	155.6	21/21
TC119041	α-1,4 glucan phosphorylase L-1	27/26	55/244	0.000	140	104.4	35/31
TC119041	α-1,4 glucan phosphorylase L-1	18/16	37/107	0.000	105	104.4	32/29
TC119364	1,4α-glucan branching enzyme Q-enzyme (EC 2.4.1.18)	13/13	25/55	0.000	105	96.6	18/20
TC112595	lipoxygenase	11/12	26/86	0.003	100	96.8	20/22
TC111732	elongation factor EF-2	13/14	26/71	0.001	95	94.2	22/24
TC121585	nematode resistance-like protein	9/10	19/32	0.009	95	129.1	11/12
TC118924	Hsp90	11/14	24/78	0.001	70	80.2	27/27
TC118997	transketolase 1 (EC 2.2.1.1)	10/10	22/63	0.001	70	73.2	23/23
TC112373	4α-glucanotransferase D enzyme (EC 2.4.1.25)	15/17	29/197	0.000	62	59.2	28/28
TC112373	4α-glucanotransferase D enzyme	14/15	27/162	0.000	60	59.2	26/26
TC132622	blight resistance protein SH20	8/8	16/75	0.005	58	105.7	13/13
TC112200	neutral leucine aminopeptidase (EC 3.4.11.1)	16/17	36/200	0.000	50	55.8	34/35
DQ114421	patatin, pat3-k1	10/11	21/136	0.000	45	40.0	26/30
DQ114417	patatin, pat2-k1	9/9	18/108	0.001	42	40.0	25/25
TC126340	succinyl-CoA ligase α subunit (EC 6.2.1.5)	4/4	8/44	0.020	37	31.2	17/17
		Fraction V					
TC112107	9–13 lipoxygenase	19/19	39/153	0.000	99	96.9	31/31
TC112465	lipoxygenase-2	10/12	23/114	0.000	98	96.9	18/21
TC112595	lipoxygenase	14/15	30/139	0.000	90	96.8	25/27
TC119108	methionine synthase (EC 2.1.1.14)	9/9	18/98	0.004	80	84.6	16/17
TC112595	lipoxygenase	13/14	28/130	0.000	75	96.8	20/20
TC112595	lipoxygenase	11/11	24/101	0.000	68	96.8	19/19
TC112026	enolase (EC 4.2.1.11)	8/9	18/119	0.001	60	47.8	36/40
DQ114421	patatin, pat3-k1 (pat1-k1, pat1-k2, pat2-k1)	6/9	12/64	0.045	42	40.0	28/37
DQ114417	patatin, pat2-k1 (pat1-k1, pat2-k1, pat2-k3)	5/8	13/51	0.030	40	40.0	25/36
DQ114417	patatin, pat2-k1 (pat1-k1, pat1-2)	5/8	11/55	0.007	39	40.0	29/38
TC119057	annexin p34	8/8	17/44	0.008	33	35.8	29/29
		Fraction VI					
TC112798	9-lipoxygenase	14/20	33/142	0.000	98	97.3	21/24
TC112026	enolase	11/14	24/172	0.001	57	47.8	42/53
TC112026	enolase	10/12	22/147	0.002	56	47.8	38/46
DQ114417	patatin, pat2-k1	10/11	23/150	0.034	40	40.0	38/38
DQ114416	patatin, pat1-k2 (pat1-k1)	6/8	14/66	0.041	42	40.0	17/19
BG350049	KPI C-k1	4[b]			25	20.2	32[b]
BG350725	KPI C-k2	4[b]			25	20.0	31[b]

Table 2. (Continued)

accession no.	protein function	peptides (no.) VEMS/ Mascot	score VEMS/ Mascot	signifi-cance VEMS	M_r SDS-PAGE (kDa)	M_r calcd (kDa)	coverage (%) VEMS/ Mascot
			Fraction VI (Continued)				
DQ207847	KPI A-k2 (KPI A-k1)	7/8	14/94	0.024	24	20.8	51/51
DQ168319	KPI B-k1	7[b]			20	20.4	49[b]
DQ168331	KPI B-k2	6[b]			20	20.3	48[b]
DQ268836	KPI B-k3	5/6	10/82	0.000	18	20.0	52/52
DQ168322	protease inhibitor I, PI I-k1	3[b]			12	9.3	34[b]
			Fraction VII				
DQ168311	KPI A-k1	6/7	13/96	0.001	25	20.8	49/49
DQ168311	KPI A-k1	7[b]			24	20.8	41[b]
DQ207847	KPI A-k2	6[b]			24	20.8	37[b]
DQ168319	KPI B-k1	8[b]			24	20.4	56[b]
DQ168331	KPI B-k2	7[b]			24	20.3	52[b]
TC111942	KPI B-k4	9[b]			24	20.0	69[b]
BG350049	KPI C-k1	6[b]			24	20.2	40[b]
BG350725	KPI C-k2	4[b]			24	20.0	31[b]
BG351756	KPI C-k3	3[b]			24	20.0	32[b]
TC119013	KPI C-k4	6[b]			24	20.2	57[b]
TC112888	KPI K-k1	2[b]			24	20.1	25[b]
TC112274	KPI M-k1	4[b]			24	21.2	18[b]
TC112554	KPI M-k2	3[b]			24	21.7	16[b]
DQ168319	KPI B-k1	7[b]			20	20.4	49[b]
DQ168331	KPI B-k2	7[b]			20	20.3	52[b]
BG350049	KPI C-k1	4[b]			20	20.2	23[b]
TC112274	KPI M-k1	4[b]			20	21.2	18[b]
			Fraction VIII				
TC112013	KPI C-k4 (KPI C-k1)	5/5	12/81	0.004	25	20.2	46/46
K1-01898	KPI C-k7	5/5	39/65	0.009	22	20.2	35/35
TC119261	carboxypeptidase inhibitor, CPI-k2	2[b]			8	4.6	54[b]

[a] Proteins with the same SDS-PAGE M_r value (column 6) were identified in the same band. One protein was in some cases identified in neighboring bands. EC number is indicated at the first identification of an enzyme only. Minor proteins identified are shown in parentheses; data relate to the major protein. Proteins were assigned to translated full-length cDNA sequences and tentative contigs (TC, www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=potato; K, www.bio.aau.dk/en/st-data.htm). DQ, BG, and the TC112888 accessions are derived from the Kuras cultivar, and Kunitz protease inhibitors (KPI) and patatins (pat) have sequences specific to Kuras (indicated by -k1, -k2, etc., in their names) (*8*). The similar PI I nomenclature is from M. Jørgensen and K. G. Welinder (unpublished data). Details are given in Supporting Information Table S1. [b] Manually identified peptides; at least one unique peptide was assigned to the protein.

an M_r at 42 kDa of the native enzyme. In Kuras glyoxalase I was previously identified at 38 kDa, p*I* 4.6, after 2D gel electrophoretic separation of juice protein (*8*), thus indicating that the potato enzyme is a monomer, in contrast to the interpretation of molecular data for soybean glyoxalase I, which was proposed to be a dimer (*38*). Therefore, the native state of glyoxalase I might vary among higher plants. The bacterial and yeast forms of glyoxalase I are monomers, whereas the human enzyme appears to be a homodimer (*39*). The highest activity of glyoxalase II was found in fraction VII, indicating an M_r near 21 kDa. Its presence was not confirmed by molecular data. The TC120743 translated into a protein of 28 kDa, suggesting that potato glyoxalase II is a monomer, similar to the 27.8 kDa *Arabidopsis* glyoxalase II (*40*). Glyoxalase I catalyzes the first step of the glyoxal pathway combining glutathione and methylglyoxal to (*R*)-*S*-lactoglutathione, which is then converted by glyoxalase II to lactic acid. The high activity found of both glyoxalase I and II in fraction I might indicate that these enzymes function in a multifunctional complex.

Fraction VII. Fraction VII is completely dominated by KPI variants eluting with M_r near 18 kDa, or 20−21 kDa as shown by MS (**Figures 1**−**3**). These monomeric proteins have pronounced inhibitory activity against trypsin and *Fusarium* protease (**Table 1**). Peptides originating from a total of 14 different KPI variants were identified and manually curated (**Table 2**; Supporting Information Table S1). The nomenclature of refs *8* and *12* is used. The present analyses confirmed that KPI variant sequences are cultivar specific (*8*). Similar KPI masses and trypsin inhibitory activity were found previously in the tubers of cv. Elkana (*2*, *4*).

MALDI-TOF MS showed additional minor peaks at 12.5 kDa, which fits the mass of protease inhibitor II (PI II) (*2*). SDS-PAGE and PMF analysis could not verify the presence of PI II, indicating that it is a minor component in Kuras tubers at harvest, in contrast to the group of PI I. Recent examples of mistaken protein assignments of KPI and PI II are Park et al. (*41*), where a PI II was assigned as KPI, and Pouvreau et al. (*4*), which assigned a KPI as "PI-2" (H. V. Nielsen and K. G. Welinder, unpublished data).

Fraction VIII. The first major peak of fraction VIII contained small molecules such as phenolics and salts as indicated by the absorbance at 310 nm and conductivity (**Figure 1**). This peak was greatly reduced by an initial concentration of the tuber juice by dry Sephadex G25, which absorbs water and molecules <3 kDa during swelling (data not shown). The last peaks within fraction VIII, eluting after salt, included proteins retained by the Superdex 200 column by unspecific adsorption.

Because small proteins give rise to very few tryptic peptides, peptide mass fingerprinting rarely provides significant protein identification. However, one carboxypeptidase inhibitor (CPI) variant was identified (**Table 2**) and a family of CPIs indicated by MALDI-TOF MS peaks at 4.0−4.6 kDa (**Figure 3**), in agreement with ref *4*. Inhibition of carboxypeptidase was not tested in the present study. MS showed two more groups of mass peaks at 5.5−5.9 and 7.6−8.2 kDa (also seen in fraction

VII), indicating the presence of various defensins, that is, snakins and thionins (M. Jørgensen and K. G. Welinder, unpublished data).

Protein Abundance in Kuras Tubers. Superdex 200 gel filtration of gently concentrated potato juice gave an excellent fractionation of functional proteins ranging from large complexes >1800 kDa to small proteins down to 4.5 kDa, thus complementing a previous 2D gel high-resolution study of Kuras tuber protein (*8*) in which the large and small proteins were lost and overcoming the dominance of patatins and KPI over less abundant proteins. The native sizes and molecular properties were determined for a number of starch biosynthetic and other enzymes, several for the first time. We observed that large protein complexes such as ribosomes, proteasomes, and chaperonin Cpn60 might be enriched by combining filtering and size exclusion chromatography, which is of potential importance to the biochemist.

The protein content of fractions was estimated by BCA and absorbance, assuming that an absorbance difference of $A_{280nm} - A_{310nm}$ of 1.0 corresponds to 1 mg of protein/mL (**Table 1**). Extinction coefficients calculated from the amino acid sequences of patatin and KPI variants were close to this value, giving 0.83−0.94 mg of patatin/mL and 0.85−1.37 mg of KPI/mL. Phenolic compounds, mostly in fraction VIII, will interfere with all methods of protein quantification, except for amino acid analysis. Subunits of larger cellular particles were identified in fractions I and II (**Table 2**), however, in minute amount. Fraction I included unspecific aggregates as judged from the high absorbance at 310 nm reflecting phenolic compounds. Protein abundance of fractions III−VII and estimated protein band intensities observed by SDS-PAGE (**Figure 2**) indicate high contents of starch phosphorylase L-1 (ca. 4% of total protein weight), mostly in fraction III, lipoxygenase (10%) and patatin variants (25%) in fraction V, PI I variants (4%) in fraction VI, and KPI variants (30%) in fraction VII, in the juice of fresh Kuras tubers at harvest. The contents of CPI and defensin variants in fraction VIII are difficult to estimate but may be near 5% each. The high content of lipoxygenases indicates that it may serve as storage protein, in addition to regulating tuber development.

Juice of the starch potato cv. Elkana, stored for 6 months, has been fractionated by Superdex 75 (*4*). This juice was reported to contain approximately 37.5% patatin, 4.5% PI I, and other proteins in peak I, 48% KPI variants (M_r 19.8−22.7, including "PI-2"), and 2% CPI. The major finding of ref *4* was that specific inhibitory activity toward different protease families followed chromatographic fractions (i.e., similar amino acid sequences) to a certain extent, however, with marked differences in specific activities as also demonstrated by ref *5*.

The study suggested that juice enzymes and inhibitors with good activities for biotechnological or food applications might be obtained by gentle protein size fractionation, whereas recombinant expression will be needed to fully characterize and produce the most potent potato juice enzymes and inhibitors for pharmaceutical applications.

ABBREVIATIONS USED

CPI, carboxypeptidase inhibitor; EST, expressed sequence tag; KPI, Kunitz protease inhibitor; LOX, lipoxygenase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; PI, protease inhibitor; PMF, peptide mass fingerprint.

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Supporting Information Available: Assignments of masses and peptides for each identified protein are given in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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