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Inhibition of Angiotensin Converting Enzyme I Caused by Autolysis of Potato Proteins by Enzymatic Activities Confined to Different Parts of the Potato Tuber

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Autolysis of protein isolates from vascular bundle and inner tuber tissues of potato (*Solanum tuberosum*) enhanced the inhibition of the angiotensin converting enzyme I (ACE), a biochemical factor affecting blood pressure (hypertension). The physiological age of the tuber affected the strength of ACE inhibition, the rate of its increase during autolysis, and the tuber tissue where ACE inhibition was most pronounced. The highest inhibitory activities (50% reduction in ACE activity achieved following autolysis at a protein concentration of 0.36 mg mL^{-1}) were measured in tubers after 5−6 months of storage prior to sprouting. The rate of ACE inhibition was positively correlated with protease activity in tuber tissues. Amendment of the autolysis reaction with protein substrates from which bioactive ACE-inhibitory peptides may be released, for example, a purified recombinant protein or a concentrate of total tuber proteins, also enhanced ACE inhibition. Many tuber proteins including aspartic protease inhibitors were degraded during autolysis. The data provide indications of differences in the enzymatic activities confined to different parts of the potato tuber at different physiological stages. Results suggest that native enzymes and substrate proteins of potato tubers can be utilized in search of dietary tools to manage elevated blood pressure.

KEYWORDS: ACE inhibition; autolysis; potato tuber (*Solanum tuberosum*); bioactive peptide; protease inhibitor

INTRODUCTION

Hypertension is a significant public health problem worldwide. It is estimated to affect one-third of the Western population and is a key factor for the development of cardiovascular diseases (*1*). One of the factors affecting blood pressure in mammals is angiotensin I converting enzyme (ACE; peptidyl-dipeptidase A, EC 3.4.15.1). ACE catalyzes the conversion of angiotensin I (decapeptide) to the potent vasoconstrictor angiotensin II (octapeptide) and also deactivation of the vasodilator nonapeptide bradykinin (*2*). On the other hand, ACE inhibitors reduce blood pressure by decreasing peripheral vascular resistance and stabilizing renal function, making them useful, for example, in reducing the progress of diabetic nephropathy (*3*). Therefore, finding new sources of ACE inhibitors especially in foodstuff is of great interest.

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ACE inhibition can be achieved with bioactive peptides that are generated during the hydrolysis of proteins. They have been studied mainly from milk, meat, and fish (*4*−*6*). Many dairy products with increased bioactive peptide contents and enhanced ACE inhibitory effects have been commercialized as functional foods targeted to consumers having elevated blood pressure (*2, 6*). However, plants have gained less attention. Those studied for ACE inhibition mostly include soybean (*Glycine max*) (*7, 8*) and pea (*Pisum sativum*) (*9, 10*). In addition, hydrolysates of a few other edible plants [wheat (*Triticum eastivum*), buckwheat (*Fagopyrum tataricum*), broccoli (*Brassica oleracea*), and rapeseed (*Brassica napus*)] also have been found to possess ACE inhibition activity (*2, 11, 12*). However, the effect of protein autolysis on ACE inhibition has not been widely studied. To our knowledge, the only studies reporting increased ACE inhibition as a result of autolysis are from the bowels of Bonito fish (*Sarda*) (*13*), shiitake mushrooms (*Lentinus edodes*) (*14*), and yeast (*Saccharomyces cerevisiae*) (*15*).

Potato (*Solanum tuberosum*) is the fourth most important food crop consumed worldwide and is known to be a source of nutritionally good quality proteins and energy (*16*−*18*). Despite

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the importance of potato for the food industry, detailed knowledge of tuber proteins and how they are modified at different physiological stages has not gained extensive interest until recently. Lehesranta et al. (*19*) reported the first comprehensive study of the potato tuber proteome with special focus on changes in the proteome during the tuber lifecycle. A few previous studies have analyzed potato transcriptome or proteome at certain stages of the tuber lifecycle (e.g., refs *20*−*24*).

We have recently found that hydrolysis of protein isolates from potato tubers results in enhanced ACE inhibition (*25*). The preliminary experiments also suggested that autolysis of tuber extracts results in higher ACE inhibition. These findings are promising in the view of the potential to enhance ACE inhibitory activity in potato by technological and biotechnological means. Autolysis offers possibilities to produce bioactive peptides without the addition of enzymes, to simplify the process, and to lower the costs. The use of autolysis also opens up a variety of possibilities to increase ACE inhibition by biotechnological tools, such as addition of protein substrates for the production of bioactive peptides. The aim of this study was to find out (i) which stage of the tuber lifecycle and (ii) which tissue of the tuber yield the highest proteolytic activity and ACE inhibitory activity during autolysis and (iii) whether enrichment of proteins in the autolysis reaction and (iv) whether the addition of bacterially expressed recombinant potato protein to the autolysis reaction enhance the production of ACE inhibitory activity.

MATERIALS AND METHODS

Plant Material. Potato cultivar Asterix was grown in a seed potato farm of Pohjoisen Kantaperuna Oy (Yppäri, Finland: latitude 64.40° north and longitude 24.09° east) in 2005 (planting, May 7) and 2006 (planting, June 11). Samples were taken at different states of potato tubers' lifecycle: (i) immature tubers 86 days postplanting (dpp) (plants at flowering stage), (ii) tubers at early maturing stage at the time of haulm killing (112 dpp), (iii) mature tubers at harvest time (133 dpp), (iv) dormant tubers after 2.5 months and (v) 5 months in storage, (vi) tubers ready to sprout following 6.5 months in storage, and (vii) the tubers stored for 6.5 months and sprouted for 14 days. In storage, the temperature was gradually lowered during the first 57 days to the proper storage temperature (3 °C, 90% relative humidity). After 6.5 months of storage, sprouting was conducted at 21−25 °C with a relative humidity of 50% in daylight for 14 days, which resulted in green vigorous sprouts of 5−10 mm in length. Sprouts were removed before taking tissue from the tubers for analysis. At each sampling time, 5 kg of tubers was taken and a subsample of 1 kg including tubers of all sizes (diameter 30−70 mm) was used for analysis. The samples were taken and analyzed similarly in both years.

Starch, Protein, and Dry Matter Content of Tubers. Dry matter was determined gravimetrically from the mixture of whole tubers of different sizes by drying samples to a constant weight. The starch content was measured spectrophotometrically by an enzymatic method (*26*), and the protein content was measured by means of the Kjeldahl method (*27*) at the Services Unit of MTT Agrifood Research, Jokioinen.

Preparation of Protein Isolates. Tubers were thoroughly washed. Proteins from potato tubers were isolated as described (*17*). The sample was comprised of tissue from the whole tuber, the peel (1 mm thick), or vascular tissue (slices of the vascular ring, 1 mm thick) excised with a knife or tuber flesh (pith) from the internal part (**Figure 1**).

Whole-tuber samples were prepared by cutting tubers into pieces of 1 cm × 1 cm, homogenizing the pieces using a laboratory blender, and adding double-distilled water at 200 mL kg^{-1} fresh weight and sodium metabisulfite (Merck, Darmstadt, Germany) at 1 g kg^{-1} fresh weight. The slurry was then stirred thoroughly. The mixed slurry was centrifuged (16300*g* for 15 min), and the supernatant was vacuum-filtered through Whatman 40 filter paper and frozen at −70 °C. Samples from different tissues of the tuber were prepared first by peeling the tuber (1 mm thick layer). The peeled tuber was cut into 1 cm thick slices, from which the vascular ring and pith (inner part) were separated with a knife (**Figure 1**). Proteins were isolated from each of the three tuber fractions. Grinding and extraction were carried out as above. Protein contents of the protein isolates were measured with DC Protein Assay (Bio-Rad Laboratories, United States) using bovine serum albumin (BSA) as a standard.

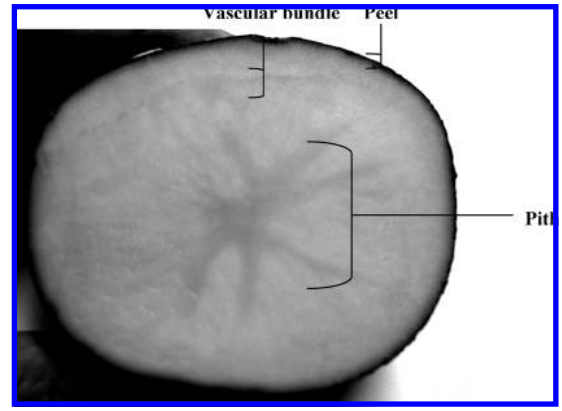

Figure 1. Peel, vascular bundle (xylem, external and internal phloem, and phloem parenchyma), and pith fractions of the potato tuber (*44, 45*) used for analysis.

Autolysis of Protein Isolates and Concentrates. Protein isolates were subjected to autolysis at a protein concentration of 8−10 mg mL^{-1}. Preliminary tests were carried out at 20, 37, 45, and 55 °C to find out the conditions for highest production of ACE inhibitory activity. Subsequently, autolysis was carried out for 2 or 5 h at 55 °C with shaking (200 rpm). Samples taken at the beginning (0 h) of autolysis were used for comparison. Samples were spun at 13000*g* for 5 min, and the clear supernatants were collected and stored at −20 °C until analysis.

The protein concentrate was prepared by subjecting a protein isolate (10 mL) to a centrifugal ultrafiltration device with the 10 kDa molecular mass cutoff membrane (Millipore, Bedford, MA). Filtration was carried out at 10 °C until the volume of the retentate was 1.0 mL (30 min, 4000*g*). The autolysis solution contained 10% (v/v) of the retentate. Autolysis was carried out at 55 °C for 5 h as described previously. Control autolysis was performed with the same nonconcentrated protein isolate.

Samples were analyzed as three triplicates, and the activity measurements were run twice from each triplicate. The protein concentration of the original protein isolate, retentate, and permeate was measured as before.

Autolysis of Protein Isolates with Added Purified Potato Protein Produced in *Escherichia coli*. Potato gene sequences predicted to encode proteins from which bioactive peptides (VPP or IPP tripeptides) (*2*) could be released were searched from Solanaceae Genomics Resource database (http://www.tigr.org/tdb/sol/). The clone TC113474 (TIGR Potato Gene Index, release 10.0) could encode a 103 amino acid long polypeptide including six VPP tripeptide sequences and was chosen for study. (During a recent update of the database, the sequence TC113474 was incorporated to the 3′-end of a 1014 nucleotide long transcript TA31025_4113 in a different open reading frame.) The gene sequence corresponding to TC113474 was cloned by extracting total RNA from sprout tissue (potato cv. Aveca) using Trizol-like reagent (*28*) followed by mRNA purification using the poly(T) columns (Qiagen, Hilden, Germany). Complementary DNA was generated by *Moloney murine leukemia virus* reverse transcriptase (Promega, Madison, WI) using a poly(T) primer. Amplification by polymerase chain reaction (PCR) was done using the forward primer 5′-GGATCCCT-TAAGATGAGCCTGACACCGCAGCTAG-3′ and the reverse primer 5′-GTCGACACGCGTGCATTTTCTTTCAACAGCCAAC-3′ (added endonuclease recognition sites are underlined). The amplification products were inserted into the pQE30 plasmid of the QIAexpress protein expression system (Qiagen) and the plasmids used to transform competent cells of *E. coli* (strain M15). Bacteria were grown overnight at 28 °C in Luria broth supplemented with 100 μg mL^{-1} carbenicillin

and 50 μg mL^{-1} kanamycin. The expressed polyhistidine-tagged protein was purified with nickel-nitrilotriacetic (Ni-NTA) beads (Qiagen) according to the manufacturer's instructions (see below). Purified protein was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and staining of the gel with Coomassie blue and also by Western blot analysis using monoclonal anti-6XHis antibodies (Amersham Biosciences, Piscataway, NJ) as described (*29*).

For the expression of larger amounts of the protein, an overnight bacterial culture was diluted 1:50 with Luria broth and grown to an optical density of 0.5−0.6. The inducer isopropyl β-D-1-thiogalactopyranoside was added to the final concentration of 1 mM, and the culture was grown further for 5 h. Cells were collected and lysed in 5 mL/g buffer B (6 M guanidine-HCl, 100 mM NaH_2PO_4, 10 mM Tris-base, and 10 mM imidazole, pH 8.0), and the protein was purified using the Ni-NTA beads. The beads were spun down and washed twice with 5 volumes of buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-base, and 10 mM imidazole, pH 6.3) according to the manufacturer's instructions. Proteins from the beads were eluted twice with 0.5 mL of buffer E (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-base, pH 4.5). The eluates were salt-exchanged to 0.075 M 2-(*N*-morpholino)-ethanesulfonic acid (MES, pH 5.7) and concentrated with centrifugal filtering (molecular mass cutoff 3 kDa; Millipore).

For autolysis, the expressed and purified protein was added to the potato protein isolate to amounts that corresponded to 2 or 5% of the total protein content of the autolysis solution. Controls included the addition of 2 or 5% of BSA or an equal volume of buffer (0.1 M MES, pH 7). Autolysis was carried out as before. Samples were analyzed as three replicates, and activity measurements were run twice from each replicate. The protein concentration was measured prior and after autolysis.

Determination of ACE Inhibition Activity by High-Pressure Liquid Chromatography (HPLC). The method of Hyun and Shin (*30*) was used to assess ACE inhibition. In the assay, 225 μL of hippuryl-L-histidyl-L-leucine (HHL) (Sigma Chemicals, St. Louis, MO) solution (5 mM in 0.1 M borate buffer, pH 8.3, containing 0.4 M NaCl) was incubated with 25 μL of sample (1 μg protein μL^{-1}) at 37 °C for 5 min, after which 75 μL of ACE solution (60 mU/mL; Sigma Chemicals) was added and incubated for 30 min. The reaction was terminated by adding 20 μL of 5 M HCl. Hippuric acid (HA) liberated by ACE was measured by RP-HPLC on a Novapak C8 column (3.9 mm × 150 mm, 4 μm; Waters, Milford, MA). The injection volume was 10 μL. The flow rate was 1 mL min^{-1} with a linear gradient (0−70% in 24 min) of acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) (Sigma Chemicals), and the effluent was monitored at 228 nm. All determinations were carried out in duplicate. The positive control for ACE inhibition (Captoril; *N*-[(*S*)-3-mercapto-2-methylpropionyl]-L-proline) and HA used as the standard for the ACE inhibition measurement were purchased from Sigma Chemicals. The ACE inhibitory activity was calculated according to the following equation: inhibitory activity (%) = $[(HA_{control} - HA_{sample})/HA_{control}] \times 100\%$. The IC_{50} value for ACE inhibition in a protein isolate was defined as the concentration of protein (mg mL^{-1}) required for 50% reduction in the amount of HA released by ACE.

Peptidase Activity Assays. The peptidase activities of the protein isolates were measured using *p*-nitroanilide amino acid substrates (Sigma Chemicals) as described (*31*). Amino acid substrates used for the determination of peptidase activities were L-leucine *p*-nitroanilide (leucine-pNA), L-lysine *p*-nitroanilide (lysine-pNA), L-glycine-proline *p*-nitroanilide (glycine-proline-pNA), and L-proline *p*-nitroanilide (proline-pNA). Samples were filtered through a 5 kDa membrane (Millipore) before analysis to remove interfering compounds such as salts and sugars. The reaction contained 50 μL of 10 mM *p*-nitroanilide substrate, 100 μL of retentate, and 100 μL of 100 mM Na-phosphate buffer (pH 7). The reaction mixture was incubated at 55 °C for 20 min. The reaction was stopped by the addition of 1 mL of 30% (v/v) acetic acid, and the absorbance was measured with a spectrophotometer at 405 nm. All enzyme activities were presented as an average of 2−4 replicate measurements. One unit of peptidase activity corresponded to the release of 1 μmol *p*-nitroanilide per minute.

Two-Dimensional (2D) Gel Electrophoresis. Protein isolates and autolysis samples were subjected to 2D gel electrophoresis as described (*32*) by loading the gel with 500 μg protein per sample. Protein samples were first solubilized in an isoelectric focusing buffer (IEFB) containing 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), a trace of Bromophenol blue, 1.5% Destreak Reagent, and 0.5% immobilized pH gradient (IPG) buffer corresponding to the pH gradient used (all reagents were from GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Isoelectric focusing was conducted with a IPGphor 3 system on IPG strips (pH 3−10, nonlinear, 7 cm; GE Healthcare Bio-Sciences AB) under paraffin oil using 8 kVh.

Before analyzing to the second dimension, the strips were equilibrated for 10 min in a buffer containing 50 mM Tris/HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of bromophenol blue, and 1% 1,4-dithiothreitol (DTT). This step enabled complete reduction of disulfide bridges and denaturation of polypeptides by SDS. A second 10 min equilibration step in the same solution containing 2.5% iodoacetamide instead of DTT was performed to block SH groups. Proteins were then subjected to SDS-PAGE. Strips were placed on the second dimension, homogeneous polyacrylamide gels (12.5% T, 1.4% C). Electrophoresis was carried out with the EttanDALTsix electrophoresis system (GE Healthcare Bio-Sciences AB) at 12 °C for 30 min at 2 W per gel and continued for 4 h at 17 W per gel.

After migration, gels were fixed and silver-stained by placing the gel into a fixative solution (30% ethanol, 0.5% acetic acid) for at least 1 h or overnight. Gels were washed with 20% ethanol and double-distilled water for 2 h and stained according to a spectrometry compatible protocol (*33*). Analysis of the same sample was done three times.

In the analysis of tuber pith fraction, the samples were purified by ultrafiltration before analysis to remove interfering compounds such as salts and sugars. Ultrafiltration was carried out by subjecting a 500 μL sample to a centrifugal ultrafiltering device with a 3 kDa molecular mass cutoff membrane (Millipore). Filtering was done at room temperature until the volume of the retentate was 200 μL (10 min, 4000*g*). An aliquot of the retentate (15 μL) was adjusted to 125 μL with IEFB as described above. Rehydration, isoelectric focusing, equilibration, second dimension SDS page, and staining were carried out as described above.

Analysis of Proteins Following Autolysis by Mass Spectrometry (MS). Proteins from 2D gel spots were analyzed at the Turku Centre for Biotechnology (Turku, Finland) using optimized procedures (courtesy Petri Kouvonen and Anne Rokka). Protein spots were picked with a scalpel and washed with NH_4HCO_3/ACN to remove impurities. After they were washed, proteins were subjected to an in-gel trypsin digestion (*34*). Peptides were desalted by a solid phase extraction with a C_{18} membrane (3M). Desalted samples were applied to an AnchorChip MALDI target plate according to the manufacturer's instructions (Bruker Daltonik, Bremen, Germany). Samples were analyzed with α-cyano-4-hydroxycinnamic acid (HCCA) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) device (Bruker Daltonik) and identified with Matrix Science Mascot Search program (Ohio Supercomputer Center, Columbus, OH). The Swiss-Prot protein knowledgebase 51.7 (http://www.expasy.org/sprot/) was used to search amino acid sequences of known proteins.

Statistical Analysis. All samples were tested in triplicate, the analyses were repeated, and experiments were carried out twice. Student's *t* test was used to compare the mean ACE inhibitory activities between treatments and samples.

RESULTS

Production of ACE Inhibition Activity during Autolysis of Protein Isolates from Different Parts of the Tuber. The protein and starch contents of the whole tubers (fresh weight) were 1−2% (6.0−7.5% of dry weight) and 14−16% (73.9−76.9% of dry weight). The mean dry matter content was 18.3−20.9%. These values were similar from the time of haulm killing until 6.5 months of storage and the subsequent sprouting for 14 days (data not shown). The small differences in means were statistically not significant ($p > 0.05$).

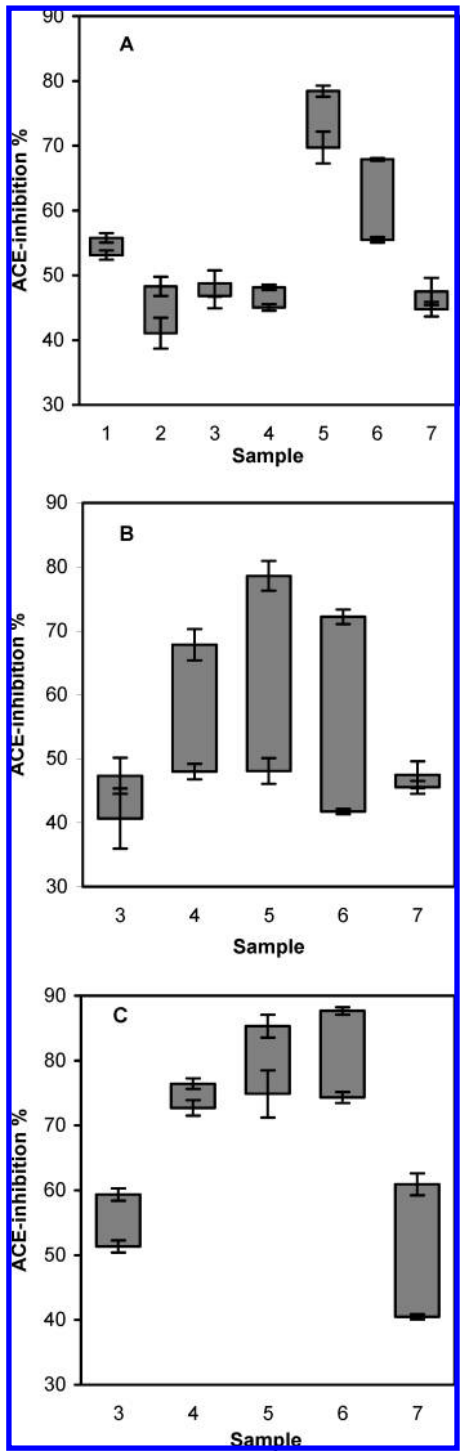

Figure 2. Increase of ACE inhibitory activity following autolysis of protein isolates prepared from (**A**) whole tubers, (**B**) pith fractions, and (**C**) vascular tissue-enriched fractions. The height of the thick bar indicates the change observed during autolysis for 5 h. The lower edge of the bar shows the ACE inhibitory activity present in the protein isolate prior to autolysis, whereas the upper edge indicates ACE inhibition level after autolysis. Thin bars indicate standard error (three autolysis reactions with two replicates; $n = 6$). Tubers were sampled at different stages of the tuber lifecycle (physiological age): 1, immature tubers ("early potatoes"); 2, haulm killing time; 3, harvest time (lifting); 4, 2.5 months of storage; 5, 5 months of storage; 6, 6.5 months of storage; and 7, 6.5 months of strorage followed by sprouting for 14 days.

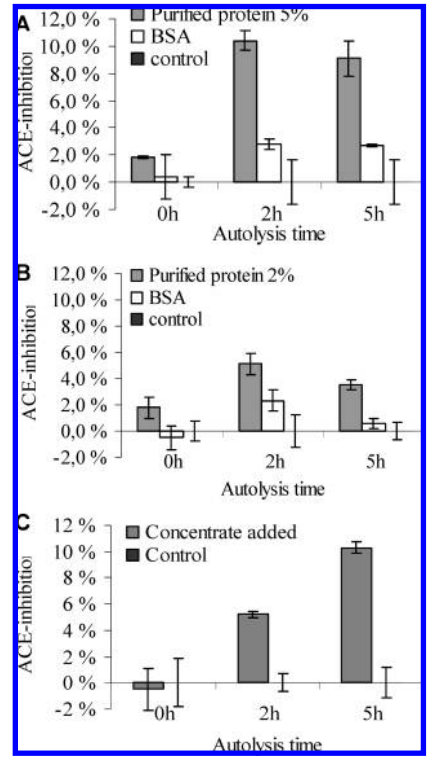

Figure 3. ACE inhibition activity increases during autolysis of protein isolates following addition of (**A**) 5 or (**B**) 2% (w/v) of purified recombinant protein or (**C**) 10% (w/w) of tuber protein concentrate prepared from the pith fraction (proteins > 10 kDa). The autolysis reaction to which proteins were added was prepared from the pith fraction of tubers stored for 6.5 months. The same sample was used to prepare the protein concentrate that was added in panel **C**. The purified recombinant protein contained six repeats of the sequence for the bioactive VPP tripeptide. BSA was added to the autolysis reaction for comparison in panels **A** and **B**. Buffer was added as the control, and the relative changes in ACE inhibition activity were compared to this control sample. Thin bars indicate standard error (three autolysis reactions with two replicates; $n = 6$).

The changes in ACE inhibition activity over time were analyzed using protein isolates from whole tubers, vascular tissue, and tissue from the inner part of the tuber designated as pith (**Figure 1**). The increase of ACE inhibition during 2 h of autolysis was consistent albeit less than following autolysis for 5 h (data not shown). Therefore, the data from 5 h autolysis only are reported here. The results from a mixture of tubers of different sizes (30−70 mm in diameter) were not significantly different from those obtained with tubers of 40 or 60 mm in diameter ($p > 0.05$; data not shown); therefore, results from tuber samples consisting of a typical range of marketable-sized tubers (30−70 mm) are presented.

Analysis of protein isolates from whole tubers prior to autolysis (**Figure 2A**, lower edge of the bars) indicated that the level of ACE inhibition in immature "early potatoes" (sample 1) and potatoes stored for 5 (sample 5) or 6.5 months (sample 6) was the highest among tubers sampled from different stages of the tuber lifecycle. However, following 14 days of sprouting, the tubers stored for 6.5 months had lost a significant portion of their ACE inhibition activity (sample 7). The inhibition activities had returned to the same lower level that was observed with protein isolates from tubers sampled at the time of haulm killing, harvesting, and following 2.5 months of storage (samples 2−4, respectively). However, following 5 h of autolysis (**Figure 2A**, upper edge of the bars), ACE inhibition showed the greatest and statistically significant increase ($p < 0.05$) in tubers sampled at the haulm killing (6.90%) and after 5 (9.2%) and 6.5 months (12.4%) of storage (**Figure 2A**). In the protein isolates from

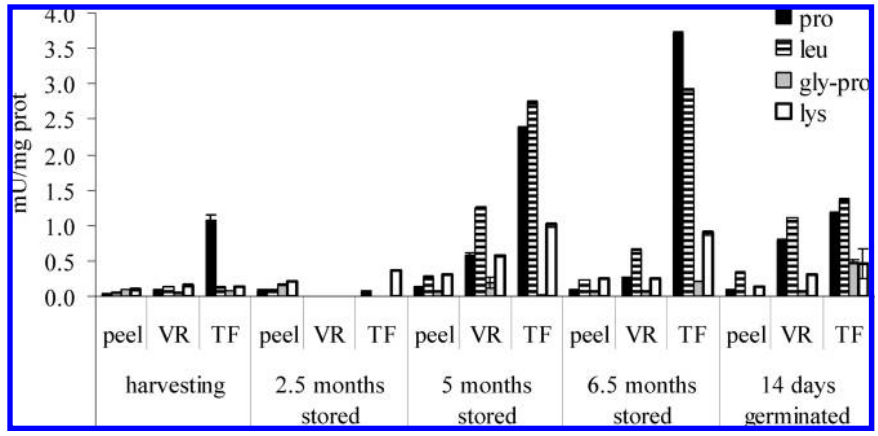

Figure 4. Protease activities in the protein isolates prepared from the peel, vascular tissue-enriched (VR), and pith fractions (TF) of tubers at different physiological ages. L-Proline *p*-nitroanilide (pro), L-leucine *p*-nitroanilide (leu), L-glycine-proline *p*-nitroanilide (gly pro), and L-lysine *p*-nitroanilide (lys) were used as substrates. One unit of peptidase activity corresponds to the release of 1 μmol *p*-nitroanilide per minute.

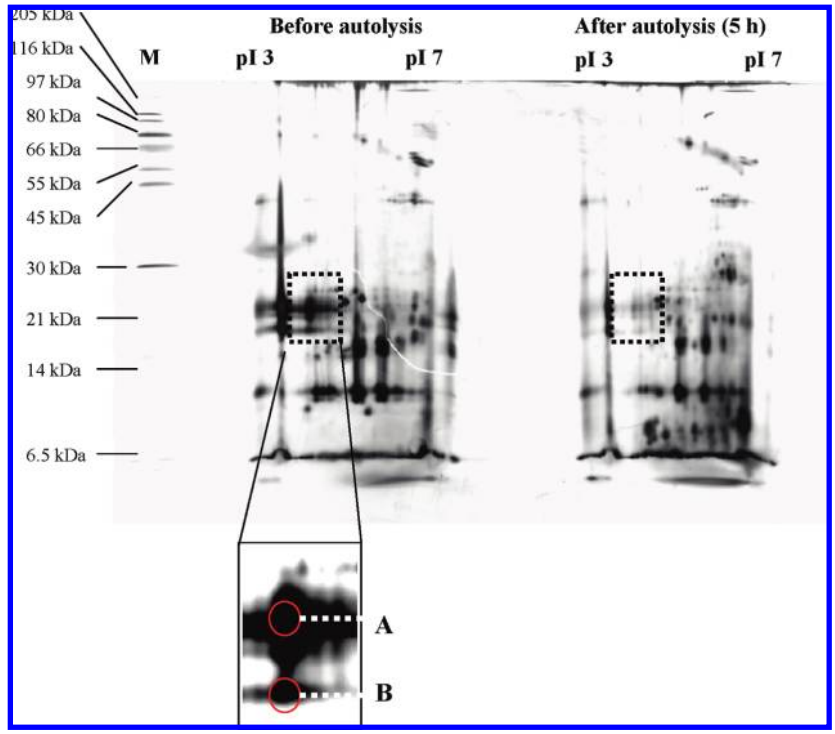

Figure 5. Analysis of protein isolates (500 μg of protein) by 2D electrophoresis before and after autolysis (5 h). The protein isolate was prepared from the pith of tubers stored for 6.5 months. The proteins in the marked area were noticeably degraded during autolysis. They were isolated from the gel, analyzed by MALDI-TOF/TOF (see **Table 1**), and identified as aspartic proteases. M, protein size marker.

"early potatoes" and the tuber samples at harvest, following 2.5 months of storage and following sprouting, no significant increase in ACE inhibition was observed during autolysis.

Subsequently, protein isolates from the pith fractions of tubers was analyzed from tubers sampled at harvest, following 2.5, 5, or 6.5 months of storage and following sprouting (**Figure 2B**). In all of these samples, ACE inhibition activities (40−48%) were similar prior to autolysis. During autolysis, ACE inhibition increased significantly ($p < 0.05$) in protein isolates from the pith tissue of tubers stored for 2.5 (19.8%), 5 (31.5%), or 6.5 months (30.4%), in contrast to tubers sampled at harvest or after sprouting, which showed no significant increase of ACE inhibition. Calculation of the IC_{50} value takes into consideration the slight differences in protein concentrations (8−10 mg mL^{-1}) between protein isolates. When it was used to indicate the rate of ACE inhibition, the pith tissue of tubers stored for 5 months had an estimated IC_{50} of 0.54 and 0.36 mg mL^{-1} prior to and after autolysis, respectively.

Samples enriched for the vascular tissue of tubers stored for 5 or 6.5 months showed the highest prior-to-autolysis ACE inhibition activities observed in this study, followed by the samples from tubers stored for 2.5 months (**Figure 2C**). However, ACE inhibition in these samples was not greatly increased during autolysis, in contrast to the pith fractions obtained from the same tubers (compare **Figure 2C** to **B**, respectively). Nevertheless, significant increases in ACE inhibition during autolysis were observed in the vascular tissue-enriched samples taken at harvest (8.0%), after 6.5 months of storage (13.4%), and after sprouting (20.4%) (**Figure 2C**).

Peels were also analyzed and found to possess high levels of ACE inhibition activity (85 ± 5%), which, however, did not change during autolysis for 5 h (data not shown). Inhibition by

Table 1. MALDI-TOF-TOF Analysis of Peptides Used to Identify Potato Tuber Proteins That Were Degraded during Autolysis[a]

	peptide mass (Da)			ion		
	observed	expected	calculated	score	rank	peptide
Protein A	1518.74	1517.73	1517.66	72	1	K.SPNSDAPCPDGVFR.Y
	2035.06	2034.05	2033.96	23	1	R.TMLLETGGTIGQADSSYFK.I+Oxidation(M)
Protein B	1518.68	1517.68	1517.66	68	1	K.SPNSDAPCPDGVFR.Y
	1348.66	1347.66	1347.64	55	1	R.YNSDVGPSGTPVR.F
	1053.56	1052.55	1052.54	24	1	K.VGNLNAYFR.T
	1969.1	1968.1	1968.07	5	1	R.RLALVNENPLDVLFQEV.-

Protein sequences[b]

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P58519   PSESPVPKPVLDTNGKELNPNSSYRIISIGRGALGGDVYLGKSPNSDAPC
P58518   --------------------NSSYRIISIGRGALGGDVYLGKSPNSDAPC

P58519   DGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIPTVKLCVSYTIW
P58518   DGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIPTVKLCVSYTIW

P58519   KVGNLNAHLRTMLLETGGTIGQADSSYFKIVKSSKFGYNLLYCPITRHFL
P58518   KVGNLNAYFRTMLLETGGTIGQADNSYFKIVKSSKIGYNLLSCPFTSIIC

P58519   CPFCRDDNFCAKVGVVIQNGKRRLALVNENPLDVLFQEV
P58518   LRCPEDQ-FCAKVGVVIQNGKRRLALVNENPLDVLFQEV
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[a] Proteins A and B correspond to spots A and B shown in **Figure 5**. MASCOT MS/MS ion search results from analysis of the trypsin-cleaved proteins using the following settings: fixed modification, carbamitomethyl (C); variable modifications; oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±0.2 Da; fragment mass tolerance, ±0.6 Da; and maximum missed cleavages, 1. [b] Sequences of proteins A (P58519) and B (P58518) detected in the SWISSPROT 51.7 database based on the peptide sequences: P58519, aspartic protease inhibitor 5 precursor (*S. tuberosum*); P58518, protease inhibitor 3 (fragment) (*S. tuberosum*). Amino acids corresponding to the peptides determined by MALDI-TOF-TOF analysis are underlined. Amino acid differences between the two proteins are shaded.

the peel fraction was similar in tubers irrespective of the physiological age (data not presented). These results suggested a role of nonprotein-based factors in ACE inhibition in the peel, which affected similarly all of the whole tuber samples analyzed.

Effect of Protein Enrichment on ACE Inhibition during Autolysis. The protein isolates prepared from pith fraction of tubers stored for 6.5 months were amended with a recombinant purified protein that was predicted to act as a source of up to six bioactive tripeptides (VPP). The added recombinant protein corresponded to 2 or 5% of the total protein amount in the sample. Data were normalized using results from the nonamended protein isolate (increase of ACE inhibition was set to 0% during autolysis) to detect the possible additional increase of ACE inhibition in autolysis that resulted from the addition of the proteins. Addition of the purified protein (5%) resulted in a significant increase (10.4%; $p < 0.01$) in the ACE inhibition activity after 2 h of autolysis (**Figure 3A**). An increase albeit less pronounced was also observed when the purified protein was added to 2% (**Figure 3B**). BSA added as a control increased the ACE inhibition only slightly. Enhancement of ACE inhibition was observed within the first 2 h of autolysis, and no further increase was observed during autolysis for up to 5 h (**Figure 3A**,**B**).

The addition of 10% (v/v) protein concentrate (>10 kDa fraction) prepared from the pith tissue sample that was also used to set up the autolysis reaction enhanced ACE inhibition during autolysis (**Figure 3C**). Enhancement of ACE inhibition lasted a longer time and developed linearly as the function of time until the end of 5 h of autolysis. At the end, ACE inhibition activity of the sample with added protein concentrate was 10% higher and significantly different ($p < 0.01$) from the autolysis reaction without protein amendment.

Peptidase Activities of Protein Isolates. The internal part (pith) of the tuber was found to show the highest peptidase activity (**Figure 4**). Following 6.5 months of storage, the peptidase activity was 3.71 and 2.92 mU mg^{-1} protein using proline-pNA and leucine-pNA substrates, respectively. Activities were correlated with tuber age (dormancy) and were lowest in dormant tubers at harvest and after 2.5 months of storage (**Figure 4**). At harvest, the pith only showed significant peptidase activity with the substrate proline-pNA (1.08 mU mg^{-1} protein). Following further storage, the peptidase activities increased, which was observed using leucine-pNA, proline-pNA, and lysine-pNA as substrates. At these stages of the tuber lifecycle, also the vascular tissue-enriched fractions showed significant peptidase activity (0.66 and 0.26 mU mg^{-1} protein using leucine-pNA and proline-pNA as substrates, respectively) (**Figure 4**). The peptidase activity in vascular tissues further increased after sprouting and was 1.12 and 0.79 mU mg^{-1} protein using leucine-pNA and proline-pNA as substrates, respectively. In contrast, peptidase activities in the pith fraction decreased following sprouting. The peptidase activity in peels was negligible, no matter at which stage of the tuber lifecycle samples were taken. These results were well-correlated with the observed increase of ACE inhibition activity during autolysis of protein isolates (**Figure 2**).

Identification of Proteins Digested during Autolysis. Proteins were analyzed by 2D electrophoresis in the protein isolates from pith fraction before and after autolysis (5 h) to detect putative sources of bioactive peptides associated with the observed ACE inhibition. Many protein spots were observed to decrease or disappear during 5 h of autolysis (**Figure 5**). A group of proteins that corresponded approximately to 20 kDa and was very pronounced prior to autolysis was hardly detectable after 5 h of autolysis (**Figure 5**). The proteins from two

spots were isolated, analyzed with a MALDI-TOF/TOF mass spectrometer, and identified. The lower molecular mass protein (**Figure 5**, spot B) was identified as a potato aspartic protease inhibitor of 18.6 kDa (Swiss-Prot protein accession no. P58518) (**Table 1**). The two peptides from the higher molecular mass protein (**Figure 5**, spot A) matched with the aforementioned potato aspartic protease inhibitor and also with a 24.8 kDa potato aspartic protease inhibitor (P58519) (**Table 1**).

DISCUSSION

Peptides inhibiting ACE have been detected in many food protein hydrolysates and ferments (*34, 25, 26, 38*), but autolysis has not been widely studied as a method to produce ACE inhibitory peptides. ACE inhibition has been reported to develop during autolysis of the bowels of bonito fish (*13, 39*), shiitake (*14*), and yeast (*15*). ACE inhibition during autolysis of potato protein isolates observed in the present study was of similar efficiency with those reported in autolysing and fermenting shiitake (*14*) and during hydrolysis of soybean protein and wheat gluten with a protease of soybean (protease D3) (*40*). While higher ACE inhibitory activities than those observed in this study may be achieved by application of commercially available proteases to protein isolates (*25*), the ACE inhibitory activities developing in potato protein isolates without addition of proteases are also remarkable according to the present study.

The production of ACE inhibitory activity varied between the different stages of the tuber lifecycle in different parts of the tuber. At the harvest time, a relatively low level of ACE inhibition activity and limited increase during autolysis were consistently observed in protein isolates prepared from whole tubers, pith tissue, and the vascular tissue-enriched fraction of tuber flesh. After 2.5 months of storage, the ACE inhibition activity as determined at the level of whole tubers had not changed and showed no significant increase during autolysis. However, the pith tissue responded to autolysis with enhanced ACE inhibition. After 2.5 months of storage and further on until 6.5 months, protein isolates from vascular tissues showed a greatly elevated ACE inhibition level, which did not increase during autolysis, whereas in samples prepared from the pith tissue, the enhancement of ACE inhibition was pronounced during autolysis. After sprouting, the protein isolates from vascular tissue-enriched samples responded to autolysis with increased ACE inhibition, in contrast to the other samples. These data suggest that the bulk of activity resulting in enhanced ACE inhibition is largely confined to the internal part of the tuber consisting of inner phloem and pith tissue.

The production of ACE inhibitory activity showed positive correlation with the observed proteolytic activity. Purified "synthetic" protein (recombinant potato protein predicted to produce seven bioactive VPP tripeptides per molecule) was added to the autolysis reaction as a putative source for release of bioactive ACE-inhibitory tripeptides and found to enhance ACE inhibition during autolysis. As little as 2−5% of the "synthetic" protein in the total protein content caused a significant increase in ACE inhibition. A similar albeit slower enhancement of ACE inhibition was achieved with a tuber protein concentrate added to the autolysis reaction. It is therefore conceivable that the observed increase of ACE inhibition was due to bioactive peptides released from proteins during autolysis. However, other substances such as trace amounts of sugars or salts in the protein isolates could also play a minor role in inhibition of ACE.

While protein isolates from whole tubers stored for 5−6 months possessed a high ACE inhibition activity, it did not increase to the same rate as did ACE inhibition during autolysis of protein isolates prepared from the inner parts of the tubers. In the samples enriched with vascular tissue, an increase of ACE inhibition during autolysis was observed only at the advent of sprouting after 6.5 months of storage and after sprouting. In peel fraction, no proteolytic activity and no increased ACE inhibition during autolysis were observed. Therefore, the data suggest that peeling potatoes or storing them until the break of dormancy does not lower their value as a source of ACE-inhibitory biopeptides. Furthermore, the findings that the compounds which produce ACE inhibitory activity during autolysis are concentrated in particular parts of the tubers offer potential for developing potato products with enhanced ACE inhibitory properties.

An aspartic protease inhibitor rich in leucine and proline was found to degrade during autolysis. Actually, two proteins were analyzed, and their different electrophoretic mobilities suggest that two aspartic protease inhibitors could be involved. However, peptide mapping could not resolve the question. It is also possible that the lower molecular mass protein resulted from cleavage of the N terminus from the higher molecular mass protein. The identified protease inhibitor(s) may be important substrates for release of ACE-inhibitory peptides because results on peptidase activity showed that the samples which most efficiently enhanced inhibition of ACE during autolysis also had the highest peptidase activities on proline-pNA and leucine-pNA substrates. Proline at the C terminus of peptides seems to be particularly effective in increasing the ACE inhibitory potential (*38*). Potato tubers contain a variety of protease inhibitors (*19*). They can constitute up to 50% of the total tuber proteins (*41*), which makes them a very potential substrate for release of ACE-inhibitory peptides. During aging, protease inhibitors are degraded in the tuber, and protease activities are consequently increased (*42*). These changes are accelerated after 6 months of storage, as shown by analysis of whole tubers (*42*). The present study showed that in tubers stored for 5−6.5 months, autolysis of the pith fraction and subsequently the vascular tissue-enhanced fraction showed a higher rate of ACE inhibition than observed by analysis of the whole tuber. In the vascular tissue-enriched fraction, the capacity to inhibit ACE was further increased after sprouting. It is therefore hypothesized that degradation of protease inhibitors is spatially and temporally regulated in potato tubers. Degradation seems to begin in the pith of the tuber and later proceeds in the vascular tissues. This process is probably attributable to activation and/or de novo synthesis of proteases that degrade also patatin, a major storage protein and potential antioxidant (*42*). Because protease inhibitors target different classes of proteases (*43*) and degradation of protein inhibitors in tubers occurs also nonenzymatically (*42*), there is scope for proteases to be active and use protease inhibitors as substrates for release of bioactive peptides.

Taken together, the data of this study provide indications of differences in the enzymatic activities confined to different parts of the potato tuber at different physiological stages, which has been little studied. Kumar et al. (*42*) and Lehesranta et al. (*19*) observed significant changes in the potato tuber proteome during tuber development and physiological age, but different tissues or fractions of the tuber were not compared. Qualitative and quantitative changes have been detected in protein content during the development of tubers (*20*), dormancy (*23*), and after dormancy (*21, 22*). All of these studies consistently show that the developmental stage and physiological age of potato tubers are associated with significant alterations in the protein composition of tubers. The present study shows that these changes

are also associated with differences in the production of ACE-inhibitory activity. Hence, potato tuber is a promising material for developing functional foods with ACE inhibitory activity. The ACE inhibitory activity of potato protein isolates can be enhanced by autolysis, fractioning tubers, and utilizing the differences between the physiological stages. Furthermore, there is potential to enhance the activity of tubers by biotechnological tools through the function of native enzymes and substrate proteins involved in the production of ACE inhibitory activity in potato tubers.

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