Isolation and Characterization of Patatin Isoforms

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Patatin has, so far, been considered a homogeneous group of proteins. A comparison of the isoforms in terms of structural properties or stability has not been reported. A method to obtain various isoform fractions as well as a comparison of the physicochemical properties of these pools is presented. Patatin could be separated in four isoform pools, denoted A, B, C, and D, representing 62%, 26%, 5%, and 7% of the total amount of patatin, respectively. These isoforms differed in surface charge, resulting in a different behavior on anion exchange chromatography, isoelectric focusing, native polyacrylamide gel, and capillary electrophoresis. All isoforms of the patatin family contained proteins with two molecular masses of approximately 40.3 and 41.6 kDa, respectively. The size of this difference in the molar mass (1300 Da) is on the order of one carbohydrate moiety. Despite the biochemical differences given above, no variations in the structural properties nor in the thermal conformational stability could be observed using far-ultraviolet circular dichroism, infrared, and fluorescence spectroscopy.

Keywords: *Patatin isoforms; isolation; characterization*

INTRODUCTION

Patatin accounts for 40−50% of the soluble proteins (Racusen and Foote, 1980). When present in potato fruit juice, patatin, like all other proteins, is prone to an irreversible heat precipitation. The mechanism leading to the irreversible precipitation in the applied industrial process is unknown. Knowledge on the structural properties and stability of patatin should help to establish the link between the observed precipitation and possible heat-induced structural changes. This could lead to the design of alternative processes of isolation, resulting in soluble potato protein that can be used in food or alternative applications. Patatin consists of a group of proteins encoded by two multigene families of which the Class I genes are expressed in relatively high amounts, exclusively in the tuber; Class II genes are expressed at a low level throughout the whole plant (Pikaard et al., 1987). Due to its high accumulation in the tuber, patatin is considered as a storage protein (Racusen and Foote, 1980; Rosahl et al., 1986). It has a lipid acyl hydrolase activity for both wax ester formation and lipid deacylation (Dennis and Galliard, 1974). It has been suggested that this activity may have a role in the plant defense mechanism (Racusen, 1984).

On the basis of the high degree of homology within the gene families and the identical immunological responses (Park et al., 1983; Mignery et al., 1984), patatin was considered as a group of single-protein species. A few papers have been published covering the genes encoding patatin, which also present some information on the individual isoforms (Park et al., 1983; Twell and Ooms, 1988; Stiekema et al., 1988; Höfgen and Willmitzer, 1990), but a comparison in terms of the structural properties or stability has not been reported. Furthermore, no papers describe the purification and subsequent characterization of patatin isoforms from the whole patatin family.

This paper presents a method to obtain various isoform pools as well as a comparison of the biochemical and the physicochemical properties of these isoform pools in relation to those of the patatin family. Our study addresses the question of whether patatin can be studied as a whole or if isolated isoforms need to be examined individually.

MATERIALS AND METHODS

Purification of the Patatin Family. The patatin family was isolated from the variety Bintje (*Solanum tuberosum*, harvest 1995) as described previously (Pots et al., 1998a), applying consecutively DEAE, concanavaline-A−Sepharose (con-A), and gel filtration chromatography.

Purification of Patatin Isoforms. Patatin isoforms were isolated using the procedure applied for the patatin family, which was modified after the con-A affinity chromatography step (Pots et al., 1998a). The patatin-containing fraction which eluted from the con-A column (denoted Pool II, 216 mL, 1.1 mg of protein/mL in a 30 mM Tris-HCl buffer pH 8 containing 0.5 M NaCl and 2 mM sodium azide) was diluted to 800 mL with a 30 mM Tris-HCl buffer pH 8 containing 2 mM sodium azide. Next, the diluted Pool II was applied to a Source Q column ($d \times h$: 6 × 10 cm; Pharmacia Biotech, Uppsala, Sweden), equilibrated, and run at 60 mL/min at 20 °C with the above-described buffer using an Äkta explorer 100 (Pharmacia Biotech, Uppsala, Sweden). All protein from Pool II bound to the column material was eluted using a NaCl gradient in the buffer described above applying the following

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gradient: 0−560 mL, 0 M; 560−1960 mL, 0.16 M; 1960−2240 mL, 0.16−0.18 M; 2240−3080 mL, 0.18−0.3 M; 3080−3500 mL, 0.3−0.5 M; 3500−4200 mL, 1 M NaCl. Detection was at 280 nm, and 22 mL fractions were collected. Appropriate fractions were combined in 4 pools, A−D. The fractions A, B, and D were diluted to a NaCl concentration of 0.15 M using the equilibration buffer and reapplied to the column. Fraction C represented a very small portion of the total protein and was neither purified nor characterized further. After rechromatography, fraction B was eluted as one peak with a NaCl gradient of 0.16−0.25 M in 1400 mL. Fraction D was re-eluted as two peaks with a NaCl gradient of 0.16−0.3 M in 1400 mL, which were separately reapplied to the column and eluted using the same NaCl gradient as in the previous run, resulting in single peaks (D1, D2) for each protein. Pool D1 will be denoted as Pool D or isoform D from now on, since it was shown that pool D2 contained a 22 kDa protein (MALDI-TOF MS) and was not characterized further. This 22 kDa protein was removed by gel filtration chromatography in the standard isolation procedure for the patatin family (Pots et al., 1998a). The protein in Pool A was re-eluted as a single peak using a NaCl gradient of 0.16−0.3 M in 840 mL.

Biochemical Analysis. Protein content was determined with the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4503) as a standard.

SDS−PAGE and IEF were performed with a Pharmacia Phastsystem according to the instructions of the manufacturer using gradient 8−25 and IEF 4.5−6 Phastgels, respectively. Gels were stained using Coomassie Brilliant Blue.

Lipid acyl hydrolase activities of the patatin family and isoforms A, B, and D were determined in triplicate as previously described using *p*-nitrophenyl laurate as the substrate (Pots et al., 1998a). Differences bigger than the standard deviation (generally approximately 1−3%) were considered significant.

The N-terminal sequence of the first 15 amino acids of the protein was determined according to the Edman procedure using an Applied Biosystems Protein sequencing system (Tempst and Riviere, 1989).

Matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF MS) analysis was performed on a Vision 2000 instrument (Thermo Bioanalysis, Hemel Hampstead, UK). The matrix used was a 9:1 (v/v) mixture of 10 mg/mL 2,5-dihydroxybenzoic acid in 0.1% TFA and 10 mg/mL 2-methoxy-5-hydroxybenzoic acid in ethanol. Detection was performed in the linear mode. Measurements of 1 μL of a 10 μmol of patatin/mL solution in 10 μL of matrix solution were performed in triplicate.

Capillary electrophoresis (CE) was performed of the patatin family and isoforms A, B, and D as described before (Pots et al., 1999) using a coated capillary (57 cm × 50 μm, Celect p150, Supelco, Bellefonte, PA) in a 150 mM sodium citrate buffer pH 3 containing 6 M urea and 0.05% methyl-hydroxyethyl cellulose (MHEC, E111−10248; Hoechst, Frankfurt am Main, Germany). The injection time was 10 s. The reproducibility of the CE analysis was tested with the intact protein. The standard deviation was up to 5%.

Reversed-phase HPLC (Thermo Separation Products, Fremont, CA) was performed using a Hi-pore RP 3−18 column (2.4−250 mm; cat. no. 125−12551, Biorad, Hercules, CA). Protein was injected via a 200 μL loop and eluted at 0.8 mL/min using a acetonitrile gradient in Millipore water as obtained by mixing solvent A (90% v/v Millipore water, 10% v/v acetonitrile, and 0.1% v/v trifluoroacetic acid) and B (10% v/v Millipore water, 90% v/v acetonitrile, and 0.07% v/v trifluoroacetic acid) in the following linear gradient steps: 0−45 min, 58−50% A; 45−50 min, 0% A; 50−55 min, 58% A. Detection was at 220 and 280 nm. Samples containing protein were collected manually in fractions of approximately 1 mL and analyzed by MALDI-TOF MS.

Structural Analysis. Far-ultraviolet circular dichroism (far-UV CD), tryptophan fluorescence, and Fourier transform infrared (FT-IR) spectra were recorded in 30 mM sodium phosphate pH 8 solutions containing the patatin family and isoforms A, B, and D (about 0.1 mg/mL of protein) at 20 °C as described previously (Pots et al., 1998a). Far-UV CD spectra were recorded as averages of 10 spectra. Fluorescence and FT-IR spectra were recorded as averages of 3 and 16 scans, respectively. In previous research it was shown that the accuracy of the determination of the midpoint of unfolding is within 0.1 °C (Pots et al., 1998b).

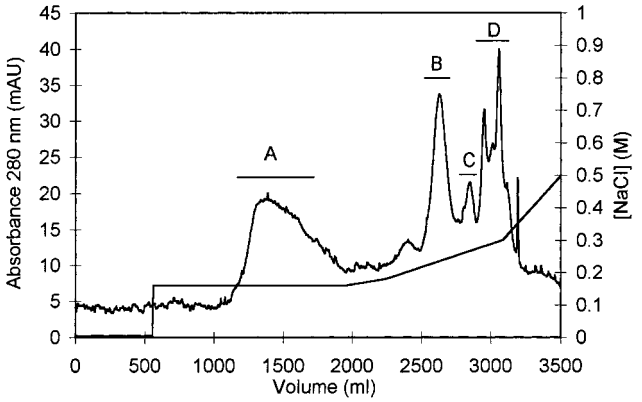

Figure 1. Elution profile of patatin isoforms (216 mL; 1.1 mg of protein/mL) on a Source-Q anion exchange column run at 60 mL/min using a NaCl gradient in a 30 mM Tris-HCl buffer pH 8 containing 2 mM sodium azide.

Table 1. Biochemical Properties of the Patatin Family and Isoforms A, B, and D

	patatin family	isoform A	isoform B	isoform D
SDS-PAGE	43 kDa	43 kDa	43 kDa	43 kDa
IEF-PAGE	6 bands pH 4.6−5.2	2 bands pH 5.0, 5.2	2 bands pH 4.6, 4.7	1 band pH 4.7
native PAGE	2 bands	1 band (upper)	1 band (lower)	1 band (lower)
MALDI-TOF MS[a]	40345 Da 41590 Da	40405 Da 41631 Da	40330 Da 41599 Da	40473 Da 41703 Da
LAH-activity[b]	3.72 ± 0.14	3.66 ± 0.08	3.55 ± 0.12	3.80 ± 0.14

[a] MALDI-TOF MS measurements were performed in triplicate. [b] Specific activity in μmol/min/mg protein ± standard deviation; measurements were performed in triplicate.

RESULTS

Fractionation into Isoform Pools. In Figure 1 the elution profile of Pool II on a Source Q column is shown. It can be seen that Pool II is fractionated into four major pools, A−D. Fraction A was eluted as a very broad peak during isocratic elution with 0.16 M NaCl. Fraction B eluted from the column with 0.16−0.18 M NaCl. Fractions C and D (two major subpeaks) eluted at 0.18 and 0.25 M NaCl. Pools A, B, and D were subsequently reapplied to the column and eluted as single peaks (results not shown) A, B, D1, and D2. As stated before, Pool C represented a very small proportion of the total protein and was not studied further and pool D2 was not patatin. Pools A, B, and D1 showed a 43 kDa band on SDS−PAGE and exhibited the lipid acyl hydrolase activity characteristic for patatin (Table 1; Galliard and Dennis, 1974; Racusen and Foote, 1980). On the basis of their contributions to the absorbance at 280 nm, the composition of the patatin family was 62% of pool A, 26% of pool B, and 5% and 7% for the isoforms C and D1, respectively. Pools A, B, and D1 will be denoted from now on as isoform A, B, and D, respectively.

Isoelectric focusing (IEF) of the patatin family showed six bands ranging from pH 4.6 to 5.2, where isoform A showed two bands, at pH 5.0 and 5.2, respectively (Table 1). Isoform B contained two bands (pH 4.6 and 4.7), and isoform D gave one band at pH 4.7. Native PAGE of

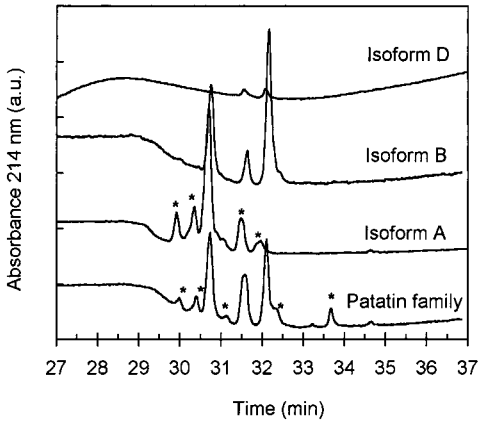

Figure 2. Electropherograms normalized for the protein concentration of the patatin family and the isoforms A, B, and D. Minor peaks are indicated with an asterisk. Samples were separated applying a voltage of 20 kV over a coated capillary (57 cm × 50 μm) in a 150 mM sodium citrate buffer pH 3 containing 6 M urea and 0.05% MHEC.

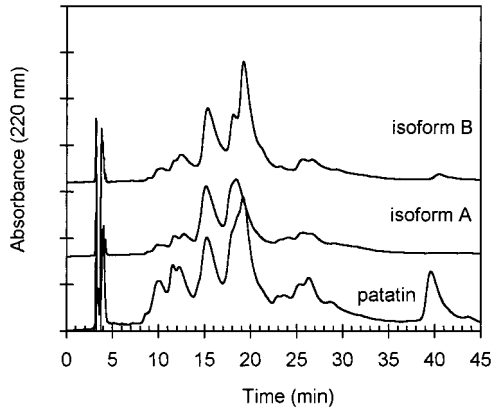

Figure 3. RP-HPLC chromatograms of the patatin family and patatin isoforms A and B on a Hi-pore 3−18 reversed-phase column. Samples of 200 μL containing approximately 0.5 mg of protein/mL were separated at 0.8 mL/min using an acetonitrile/trifluoroacetic acid gradient in Millipore water.

the patatin family, containing all isoforms, revealed two bands, whereas isoforms A, B, and D showed a single band (Table 1). Isoform A exhibited the upper band of the two observed in the patatin family, whereas isoforms B and D contained the lower band.

Biochemical Properties. A number of analytical techniques were applied to examine the differences between the isoforms. All isoforms and the patatin family showed two distinct molecular masses with MALDI-TOF MS of approximately *m*/*z* 40 390 (standard error ± 65) and 41 630 (standard error ± 50) Da, respectively (Table 1). The intensities of the peaks as observed with MALDI-TOF MS for these molecular masses were essentially the same (results not shown). No significant differences in lipid acyl hydrolase activity between the patatin family and the patatin isoforms were observed (Table 1). The N-terminal sequence of the first 15 amino acids was Thr, Leu, Glu, Glu, Met, Val, Thr, Val, Leu, Ser, Ile, Asp, Gly, Gly, Gly, and both the patatin family and the isoforms showed mutations at the first (Thr/Lys) and the third (Glu/Gly) position.

In Figure 2 the electropherograms of the patatin family are presented as well as isoforms A, B, and D, normalized for the protein content of the samples. It can be seen that the patatin family exhibits three major peaks (R_t = 30.7, 31.5, 32.1 min) and at least five minor peaks, indicated with an asterisk (R_t = 30.0, 30.4, 31.2, 32.2 [shoulder of 32.1 min major peak], 33.7 min).

Isoform A contains one major peak (R_t = 30.7 min) and four minor peaks as indicated with an asterisk (R_t = 30.0, 30.4, 31.5, and 32.0 min). Isoform B exhibits all three major peaks that are observed in the patatin family, whereas the minor peaks are virtually absent. Isoform D shows only two minor peaks at 31.5 and 32.1 min; the limited contribution of this protein fraction to the total amount of protein in the patatin family (Figure 1) obstructed further analysis of this isoform.

The patatin family could be separated into about 10 peaks using reversed-phase HPLC (RP-HPLC). Major peaks were observed around 15, 19, and 39 min (Figure 3); minor peaks eluted after 12 and 23−28 min. Isoform A exhibited two major peaks at 15 and 19 min. It showed minor peaks that seemed to be lower compared to those of the patatin family, and the peak at 38 min observed in the patatin family is absent. Isoform B showed two major peaks around 15 and 19 min. The peaks at 9−12, 22−28, and especially 39 min were lower compared to those of the patatin family, as observed for isoform A as well. The major peak at 19 min of isoform B seems to consist of two proteins, as seen from the shoulder peak. The broad peaks around 19 min of the patatin family and isoform A allow one to speculate about the presence of at least two proteins in these peaks also. The top of the major peak at 19 min of isoform A seems to be at shorter retention times than those of the patatin family and isoform B; it could be possible that isoform A contained more of the proteins, causing the shoulder peaks at 19 min in the patatin family. To investigate whether the isoform pools could be separated into mass isomers, the major peaks of the patatin family (15, 19, and 38 min) and isoforms B and A (15 and 19 min) were collected and the molecular mass of the corresponding protein was determined with MALDI-TOF MS.

Table 2. MALDI-TOF MS Analysis of RP-HPLC Fractionated Patatin Family and Isoforms[a]

sample	retention time (min)	molecular mass (Da)	
patatin family unfractionated		40 345	41590
patatin family fractionated	15	40 444	41 576[b]
	19	n.d.[c]	
	38	n.d.	
isoform A	15	n.d.	
	19	40 489	41 607
isoform B	15	40 377	41 532
	19	40 425	41 589

[a] The measurements were performed in triplicate. [b] Error 0.5%. [c] nd: no peaks could be detected.

In Table 2 molecular masses are presented for the unfractionated patatin family, the RP-HPLC fractionated patatin family, and isoforms A and B. All RP-HPLC fractions giving peaks using MALDI-TOF MS exhibited the two mass isomers, as observed for the unfractionated patatin family. The reason for the difference in molecular masses apparently does not influence the interactions of the protein with the column material, i.e., the mass isoforms are not separated using RP-HPLC. The molecular mass of the corresponding proteins of all major RP-HPLC peaks could not be established and could possibly be due to aggregation of the proteins. This aggregation could be due to the polarity and pH of the solvent (acetonitrile in water containing TFA), since the protein unfolds at acidic pH values (Pots

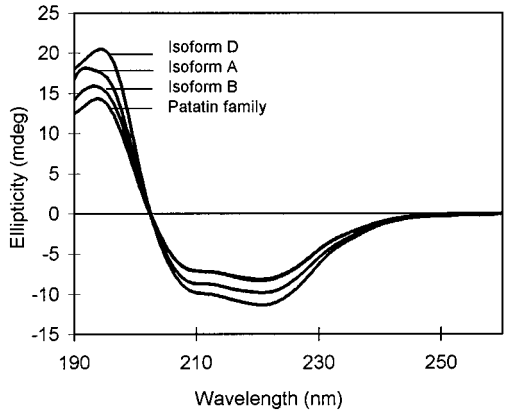

Figure 4. Far-UV CD spectra of the patatin family and isoforms A, B, and D as determined from 190 to 260 nm. The protein concentrations were 0.1 mg/mL; each curve represents an average of 10 scans.

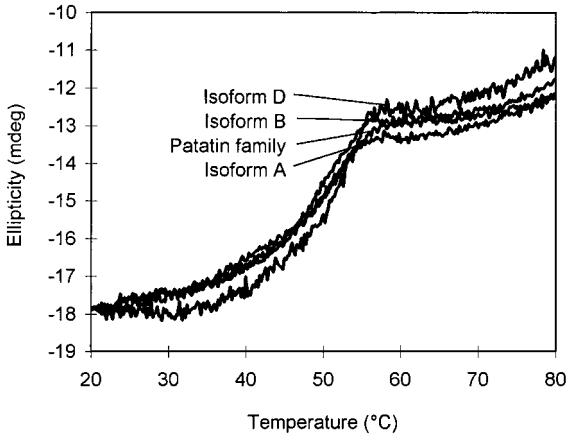

Figure 5. Thermal unfolding of the patatin family and isoforms A, B, and D as determined using far-UV CD. The ellipticity at 222 nm was measured upon heating from 20 to 80 °C at a rate of 20 °C/h. The protein concentrations were 0.1 mg/mL.

et al., 1998B). This could allow aggregation and resulted in very low responses in the MALDI-TOF mass spectrometer. These low responses caused the relatively high error in the measured molecular mass.

Structural Properties and Thermal Stability. To study possible differences in conformation and structural stability between the isoforms, far-UV CD, FT-IR, and fluorescence spectra were recorded at ambient temperature and as a function of temperature. In Figure 4, far-UV CD spectra are shown for the patatin family and isoforms A, B, and D. All spectra exhibit the same extrema at 195, 210, and 222 nm, as well as a zero crossing at 203 nm. This indicates that all isoforms have a highly identical secondary structure (Johnson, 1988). These results were confirmed with FT-IR spectroscopy. The amide I region of all isoforms showed similar shape and a maximum at about 1645 cm^{-1} (results not shown). Also, the wavelength of maximum intensity (335 nm) and the shape of the tryptophan fluorescence spectra were similar for all isoforms and the patatin family (results not shown). This implies that there are no conformational differences between the isoforms at a tertiary level of folding of the tryptophan environment (Pace et al., 1988) at ambient temperatures.

In Figure 5 is shown the ellipticity at 222 nm as a function of temperature of the patatin family and the patatin isoforms A, B, and D. The ellipticity at 222 nm is used as a measure for the α-helix content of the protein. All isoforms showed a similar unfolding pattern resulting in a midpoint of unfolding of 49 °C, as previously observed for the patatin family at pH 8 also (Pots et al., 1998b). The same unfolding temperatures were obtained with tryptophan fluorescence as a function of temperature (results not shown). Despite the charge differences between the isoforms, their conformation and structural stability appears to be similar. These mutations have no influence on the catalytic activity of patatin and neither a conformational effect nor effect on the structural stability.

DISCUSSION

The aim of this study was to obtain a procedure to isolate patatin isoforms; to answer the question of whether the individual isoforms have the same structural properties and stability as the patatin family. If so, it would be sufficient to study the physicochemical properties of the whole patatin family instead of those of the individual isoforms. The two major isoforms were 88% of the protein in the patatin family.

Charge Differences between the Isoforms. The differences between the patatin isoforms (Figures 1 and 2; Table 1) can be explained by charge differences between the proteins. Isoform A has, for example, at pH 8, the weakest interaction with the anion exchange column (Figure 1). Furthermore, it exhibits the shortest running distance on native PAGE at pH 8, at similar molecular masses as the other isoforms (Table 1). Apparently, isoform A has the lowest amount of surface charge of all isoforms. Furthermore, isoform A has the highest IEP (Table 1) and the shortest retention time on CE (at pH 3; Figure 2). This suggests that isoform A contains either relatively more residues that are positively charged at pH 3 (His, Lys) than isoforms B and D, or relatively more residues that are negatively charged (Asp, Glu, His) at pH 8 than isoforms B and D, or both. The presence of patatin isoforms with mutated charged amino acids has been described in the literature (Mignery et al., 1984; Stiekema et al., 1988). The primary sequences of two patatin isoforms from the cultivar Bintje as obtained from the cDNA sequence show one mutation which can have an effect on the net charge of the protein (mutation Asp/Asn_{355}; Stiekema et al., 1988). The isoforms from cultivar Superior showed four mutations involving charged residues (Gly/Glu_3, Val/Glu_{215}, Asn/Asp_{226}, Glu/Gln_{250}; Mignery et al., 1984). The first mutation (Gly/Glu_3) of charged amino acids was also observed in this research. So, it occurs not only in the cultivar Superior but also in the variety Bintje. Sequencing of the complete primary structure of the isoforms could establish the presence of these charge differences due to mutations. That was not performed, however, since it would be extremely time-consuming, and the behavior, structure, and the conformational stability apparently is not influenced by these charge differences. The error in the accuracy of the MALDI-TOF MS measurements was 0.5%, which did not allow assignment of the measured differences between isoforms (Table 2).

Molecular Mass Differences between Isoforms. All isoform pools were still a mixture of two mass isomers (approximately 40.4 and 41.8 kDa; Tables 1 and 2). The differences in molecular masses observed within the patatin family and the isoforms cannot likely be explained by mutations in the primary sequence. For

example, the two examined isoforms of the variety Superior vary in 21 of the 366 amino acids (94% homology; Mignery et al., 1984). When the differences in the molar masses of the mutated amino acids are summed, a difference of maximal 663 Da can be obtained. The actual calculated difference between two isoforms was found to be 100 Da (Mignery et al., 1984). The same calculations can be performed for isoforms of Bintje. These isoforms show a homology of 97.5% and can have a mass difference due to point mutations of maximal 198 Da (Stiekema et al., 1988). Even if one isoform contains all the amino acids with the lowest molecular mass, the mass difference cannot explain the mass difference observed with the MALDI-TOF MS experiments. The size of this difference (approximately 1.2 kDa) is on the order of the molecular mass of the carbohydrate antenna of the protein (Man(α1−3)[Man(α1−6)][Xyl(β1−2)]Man(β1−4)GlcNAc(β1−4)[Fuc(α1−3)]GlcNAc, mw: 1169 Da; Sonnewald et al., 1989). The latter study revealed that patatin has three possible glycosylation sites (Asn at position 60, 90, and 202) of which two were actually glycosylated in the examined isoform (Sonnewald et al., 1989). In analogy to the results found by Sonnewald et al. (1989), it could be possible that one fraction of the protein in the potato is glycosylated at one position and that the other fraction is glycosylated at two positions whereas the third is not used. The molecular mass of a patatin isoform without the carbohydrate contribution, as calculated from its primary sequence derived from the cDNA, is 39 745 (Stiekema et al., 1988) or 39 564 and 39 664 (Mignery et al., 1984) Da. Adding the mass of one, two, or three carbohydrate chains would increase the total mass to 40 914, 42 083, and 43 252 Da as observed by Stiekema et al. (1988), or 40 733, 41 902, or 43 071, and 40 833, 42 002, or 43 171 (Mignery et al., 1984), respectively. Comparing the calculated with the measured molecular masses in this study, differences are obtained of 300−500 and 100−200 Da for the low and high molecular mass isomer, respectively. These differences could be due to point mutations in the primary sequence but also to an incorrect presentation of the carbohydrate moiety in the literature (Sonnewald et al., 1989) or inaccuracy of the MALDI-TOF measurements.

Structural Properties of Isoforms. From the literature it is known that for other proteins consisting of various genetic variants (for example, β-lactoglobulin), differences in behavior between genetic variants exist, like susceptibility to proteolysis or denaturation temperature (Schmidt and van Markwijk, 1993). Patatin does not show a comparable behavior, in this research no differences between the isoform pools in terms of conformation or structural stability were observed. It has also been reported that patatin isoforms show no differences toward proteolytic breakdown in the whole potato during storage (Pots et al., 1999). Furthermore, only minor differences are observed in the substrate specificity of Class I and II patatin (Höfgen and Willmitzer, 1990).

In conclusion, the patatin family, showing 10 peaks with RP-HPLC, can be separated into four pools (A−D) based on surface charge differences. The two major pools, A and C, represent 62% and 26% of the patatin family, respectively. Within all isoform pools two molecular masses were found. Despite differences between the isoforms in surface charge and mass differences within the pools, the latter most likely due to differences in degree of glycosylation, neither differences in biochemical and structural properties nor conformational stability could be observed. The behavior of patatin can be studied without he need to examine the isoforms individually.

ABBREVIATIONS USED

MALDI-TOF MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; LAH, lipid acyl hydrolase; CE, capillary electrophoresis; CD, circular dichroism; FT-IR, Fourier transform infrared.

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