JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 6419−6427 **6419**

Effects of Protein Composition and Enzymatic Activity on Formation and Properties of Potato Protein Stabilized Emulsions

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In the present study emulsions were made with various potato protein preparations, which varied in protease inhibitor and patatin content. These emulsions were characterized with respect to average droplet size, plateau surface excess, and the occurrence of droplet aggregation. Droplet aggregation occurred only with potato protein preparations that contained a substantial amount of protease inhibitors and could be prevented only at pH 3. The average droplet size of the emulsions made with potato proteins appeared to be related to the patatin content of the preparation used. Average droplet size was found to be dominated by the patatin-catalyzed lipolytic release of surface active fatty acids and monoglycerides from the tricaprylin oil phase during the emulsification process. Addition of monoglycerides and especially fatty acids, at concentrations representative of those during emulsification, was shown to cause a stronger and much faster decrease of the interfacial tension than that with protein alone and to result in a drastic decrease in droplet size. The patatin used was shown to have a lipolytic activity of 820 units/g with emulsified tricaprylin as the substrate. Because of the droplet aggregating properties of the protease inhibitors, the patatin-rich potato preparations seem to be the most promising for food emulsion applications over a broad pH range, provided the lipolytic activity can be diminished or circumvented.

KEYWORDS: Potato protein; emulsion; potato juice; industrial starch; patatin; protease inhibitors; lipolytic activity

INTRODUCTION

Potato juice (PJ) is a byproduct from industrial starch manufacture and contains ≈1.5% (w/v) soluble protein (*1*). Potato protein has a relatively high nutritional quality (*2*, *3*) and, therefore, has good potential for utilization in foods. However, recovering the protein by heat coagulation from PJ results in a complete loss of most of its functional properties for industrial application. Several efforts have, therefore, been made to recover potato proteins from PJ that have retained their functional properties (*4*, *5*).

The potato proteins have been classified tentatively into three classes: patatin family (41 kDa; ≈40% of total soluble protein), protease inhibitors (5−25 kDa; ≈50% of total soluble protein), and others (mostly high molecular weight) (*6*). Patatin has a known lipid acyl hydrolase (LAH) activity (*7*). It shows a high activity on mono- and diacyl phospholipids, mono- and diacyl glycerols, and galactosyl diglycerides but seems to have no activity on triacyl glycerols (*7*−*10*, *12*). The crystal structure of one patatin isoform has recently been solved (*11*), and its active site was shown to consist of a Ser−Asp catalytic dyad (*8*, *11*) and is said not to be able to hydrolyze lipids in a micellar environment (*11*).

In many food products proteins are used as emulsifiers and emulsion stabilizers. The emulsifying properties of undenatured potato proteins have been studied to only a limited extent (*13*, *14*). They were reported to be inferior (*13*) or superior (*16*) to the so-called emulsifying properties of whey proteins, but superior to those of a commercial soy isolate (*15*) and casein (*16*). Most authors (*13*, *14*), however, determined parameters such as emulsifying capacity and performed empirical emulsion stability tests, which have little practical relevance (*17*).

In the study of emulsion properties a distinction should be made between formation and stabilization of the emulsion (*18*).

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10.1021/jf061278z CCC: $33.50
Published on Web 07/27/2006

During emulsion formation the presence of surfactant serves two main purposes, of which the most important is the ability to form interfacial tension gradients, which stabilize the newly formed droplets against immediate (re)coalescence. Surfactants also lower the interfacial tension (γ), thus facilitating the breakup of droplets, which is opposed by the Laplace pressure ($p_L = 4\gamma/d$) (*18*).

Once an emulsion is formed, various instabilities may occur. Creaming is the rise of droplets to the top of the emulsion due to the density difference between the dispersed phase and the continuous phase.

Droplet aggregation may also occur in emulsions, and it increases the effective average particle size and, therefore, usually results in a decreased stability of the emulsion against creaming. Besides aggregation due to net colloidal atrraction between droplets, specific mechanisms of aggregation may be observed, which are called bridging and depletion flocculation. During bridging flocculation one polymer molecule adsorbs at two interfaces, thus forming a bridge between the droplets. Depletion flocculation may occur when high concentrations of non-adsorbing polymers are present. Finally, droplet coalescence may occur during storage. The purpose of this study was to examine emulsion-forming and -stabilizing properties of various potato protein preparations under various conditions (pH, ionic strength, after heat treatment, after ethanol induced precipitation) in a more systematic and more extensive way than has been done until now. The emulsions are characterized with respect to their average droplet size, surface excess, and stability against droplet aggregation. In addition, the lipolytic activity of patatin in a tricaprylin emulsion is investigated, and the effect of this activity on the emulsion properties of emulsions made with various potato protein preparations is discussed.

MATERIALS AND METHODS

Preparation of Protein Fractions. *Potato Juice.* PJ from cv. Elkana was prepared as described previously (*19*). This PJ has a pH of 5.7−6.0 and is known to be similar to industrial PJ (AVEBE B.A., Foxhol, The Netherlands).

Potato Protein Isolate (PPI). PPI was prepared as described previously (*20*) and stored at −20 °C. PPI contains most of the proteins present in PJ. These proteins are unfolded to a limited extent due to their precipitation in the presence of ethanol, which is used during the isolation (*20*).

Ammonium Sulfate Precipitate (ASP). ASP was prepared as described previously (*19*) and can be regarded as a simulant of undenatured total potato protein. ASP was stored at −20 °C. It contains most of the proteins present in PJ.

Patatin. Patatin was purified from PJ as described previously (*19*), except that after diafiltration, the patatin was stored at 4 °C at a concentration of 20 mg/mL in 9 mM sodium phosphate buffer (pH 7), containing 0.2 g/L of sodium azide to prevent microbial growth.

Ethanol-precipitated patatin was prepared as described previously (*20*) and is denoted PAT-5E. Patatin is estimated to make up 38% of potato protein in PJ of cv. Elkana (*6*). PAT-5E is patatin that has a partly unfolded tertiary structure due to precipitation at pH 5 in the presence of ethanol (*20*).

Protease Inhibitor Pool (PIP). PIP protein was prepared from PJ as described previously (*19*) and stored at −20 °C. Ethanol-precipitated PIP (PIP-5E) was prepared as described previously (*20*).

PIP contains mainly protease inhibitors of molecular sizes between 15 and 25 kDa (*19*) and is estimated to contain 45% of the protein present in PJ of the cultivar used (*6*). PIP-5E has approximately the same protein composition as PIP but has been precipitated in the presence of ethanol, which is expected to have caused small irreversible changes in the conformation of the proteins (*20*).

Preparation of Protein Dispersions. Protein dispersions (7.5 mg/mL) at pH 7.0 were prepared from ASP, PPI, PIP, PIP-5E, patatin, and PAT-5E by dispersing these proteins in 9 mM sodium phosphate buffer (pH 7.0) containing 0.2 g/L of sodium azide, when an ionic strength of ≈15 mM was desired. When an ionic strength of 50 mM was desired, 35 mM of NaCl was added to the buffer.

Protein dispersions (7.5 mg/mL) at pH 5.0 were prepared from ASP, PPI, PIP, and patatin by dispersing these proteins in 24 mM sodium acetate buffer (pH 5.0) containing 0.2 g/L of sodium azide and 185 mM NaCl to reach an ionic strength of 200 mM.

Protein dispersions (7.5 mg/mL) at pH 3 were prepared from ASP, PPI, PIP, and patatin by dispersing these proteins in 17 mM sodium acetate buffer (pH 3) containing 0.2 g/L of sodium azide, which had an ionic strength of ≈15 mM. Parts of the protein dispersions of ASP, PPI, and patatin at pH 3 were readjusted to pH 7.0 after 1 h by the addition of 1 M NaOH, and their ionic strengths were adjusted to 50 mM by the addition of appropriate amounts of NaCl; this is referred to as the pH 3 → pH 7 treatment.

Protein samples used for testing the effect of heat treatment were prepared by making dispersions of 10 mg/mL of ASP, PPI, PIP, and patatin in 9 mM sodium phosphate buffer (pH 7.0) containing 0.2 g/L of sodium azide ($I \approx 15$ mM). The dispersions were filtered over a 0.2 μm filter (Schleicher & Schuell, Dassel, Germany) and subsequently heated in a thermostated water bath at 80 ± 1 °C for 10 min after the temperature in the sample had reached 80 °C. Heated samples were immediately cooled in ice water, and their ionic strengths were adjusted to 50 mM by the addition of NaCl, after which the dispersions were again filtered over a 0.2 μm filter.

Patatin samples in which the LAH activity of the patatin had been irreversibly inhibited by the addition of methyl arachidonyl fluorophosphonate (MFAP, Sigma) to a concentration of 27 μM (*21*) were also prepared. The activities of both the active and the inactivated patatin were measured as described previously (*19*) using 0.87 μM PNP-caprylate as a substrate.

All protein dispersions prepared were stirred overnight at 4 °C and were subsequently equilibrated at room temperature; the pH was monitored and, if necessary, adjusted with small volumes of 1 M NaOH or 0.5 M H_2SO_4. Next, the protein dispersions were centrifuged (10000*g*, 20 min, 25 °C) and subsequently filtered over a 0.2 μm filter. The protein concentration of the final dispersions was estimated using the Bradford assay (*22*) with bovine serum albumin (Sigma A-7511; lot 92H93131) as standard.

Preparation of Emulsions. Emulsions were prepared by mixing 18 mL of protein dispersion and 2 mL of tricaprylin oil (Sigma; $\rho = 950$ kg m^{-3}; $n_D = 1.4466$). To investigate the effect of lipolysis products of tricaprylin on the emulsifying properties of patatin, also oil in which monocaprylin (5 or 25 mM) or caprylic acid (25 or 100 mM) had been dissolved was used to prepare emulsions. A coarse pre-emulsion was prepared using an Ultra Turrax type T-25B (Janke & Kunkel GmbH) at 11000 rpm during 1 min. The pre-emulsion was homogenized by passing it 30 times through a Delta Instruments HU 2.0 laboratory scale high-pressure homogenizer (Delta Instruments, Drachten, The Netherlands) operated at 6 MPa. Emulsions were prepared and tested at least in duplicate.

Droplet Size Estimation. The average droplet size was calculated as the volume-surface average diameter (d_{32}) given by $d_{32} = S_3/S_2 = \Sigma N_i d_i^3/\Sigma N_i d_i^2$, with N_i and d_i the number and diameter of droplets in size class i, respectively. The relative width of the droplet size distribution is then given by $c_s = (S_2 S_4/S_3^2 - 1)^{0.5}$ (*23*), in which S_4 is $\Sigma N_i d_i^4$. The parameters mentioned were determined using the spectroturbidimetric method described by Walstra (*23*, *24*). Emulsions were prepared in duplicate, and the absorbance of the emulsions was measured for each emulsion at wavelengths from 380 to 1700 nm using a Zeiss spectrophotometer (type M 4 GII) with an attachment for turbidity measurements and an angle of acceptance of 1.5°. Before measurement, the emulsions were diluted with 0.3% (w/v) sodium dodecyl sulfate (SDS) to stabilize the droplets and disperse any aggregates present, until a final optical density between 0.2 and 0.8 was reached. The absence of aggregates was verified by light microscopy (magnification = 400×). In the experiments in which monocaprylin and capylic acid had been added to the oil d_{32} values were estimated using the Coulter LS 230 (Beckman-Coulter Inc., Fullerton, CA).

Estimation of Protein Surface Excess (Γ). The protein surface excess of emulsions was estimated using an indirect depletion method, which is based on the estimation of the amount of unadsorbed protein and the interfacial area of the emulsion (*25*). For patatin (pH 7, $I = 50$ mM) the surface excess (Γ) was determined as a function of the protein concentration over the interfacial area of the emulsion (c/A), in which c is the protein concentration (mg/m^3) and A is the specific area (m^2/m^3) of the emulsion. The specific area can be calculated from $A = 6\varphi/d_{32}$ (*26*), in which φ is the volume fraction of oil in the emulsion. For these experiments patatin concentrations ranging from 0.67 to 10 mg/mL were used, whereas for other emulsions Γ was determined at a single protein concentration, at which Γ was known to have reached a plateau value. To determine the concentration of unadsorbed protein, the emulsions were centrifuged (30 min, 14000*g*, 25 °C), resulting in a cream layer and a serum layer. About one-third of the bottom part of the serum layer was taken and centrifuged again. This procedure was repeated three times, and the final serum was filtered over a 0.2 μm filter and its protein content was estimated (c_{serum}). The cream layers obtained after the first centrifugation step were redispersed in buffer volumes equal to those in the original emulsion to obtain a washing liquid. The washing liquid obtained by centrifuging (30 min, 14000*g*, 25 °C) the redispersed emulsion was filtered over a 0.2 μm filter, and its protein content was determined when no coalescence was observed during the washing procedure ($c_{washing1}$). If coalescence was observed, a new sample of the emulsion was taken and treated as described above. The washing procedure was repeated twice ($c_{washing2}$). The protein content of the protein solutions used was estimated using the method of Bradford (*22*) with bovine serum albumin (Sigma A-7511) (lot 92H93131) as standard. The surface excess was calculated as $\Gamma = \Delta c$ (mg/m^3)/A (m^2/m^3), in which $\Delta c = c_{emulsion} - c_{serum} - c_{washing1} - c_{washing2}$ and c is the protein concentration in mg/m^3. The precision of the Γ values was estimated as described in the appendix of the paper by Oortwijn and Walstra (*25*). The average standard deviation of the average droplet size was estimated as 0.12 μm, resulting, for example, in the case of an emulsion with $d_{32} = 0.58$ μm in $\sigma(A) = 1.0$ m^2, in which $\sigma(A)$ is the standard deviation of the surface area of 1 mL of emulsified oil. The other parameters for this emulsion were estimated to be $\Delta c = 3.71$ mg/mL, $\sigma(c) = 0.065$ mg/mL, $A = 10.3$ m^2, $\varphi = 0.1$, and $\sigma(\varphi) = 0.0005$, in which $\sigma(c)$ and $\sigma(\varphi)$ are the standard deviations of Δc and φ, respectively. From these values the standard error of Γ was calculated as being 0.29Γ. Therefore, when emulsions were compared, differences in surface excess of less than ≈30% and differences in average droplet size (d_{32}) of less than 0.12 μm were considered to be not significant.

Viscosity Measurements. The viscosity of a number of emulsions was estimated as a function of shear rate (4−135 s^{-1}), using a Bohlin CVO rheometer (Bohlin Instruments, Cirencester, U.K.) at 20 °C using a finely grooved cylinder (C25) measuring body.

Interfacial Tension Measurements. Interfacial tension measurements as a function of time were performed on a tricaprylin oil droplet of ≈25 μL using axis symmetric drop-shape analysis with an Automated Drop Tensiometer (IT Concept, Longessainge, France) as described by Benjamins et al. (*27*). Oil droplets were made in a solution of patatin (4 mg/mL) in 9 mM sodium phosphate buffer (pH 7) using tricaprylin oil or tricaprylin oil containing 2, 10, or 25 mM 1-monocaprylin or 25, 50, or 100 mM caprylic acid.

Extraction and Analysis of Lipid Components. Duplicate emulsions were made from 90 mL of 5 mg/mL patatin in 9 mM sodium phosphate buffer (pH 7) and 10 mL of tricaprylin oil by making a preemulsion and immediately passing this 10 times through the homogenizer at 6.0 MPa. These emulsions were characterized with regard to their droplet size distribution, and of each emulsion a sample (1 mL) was taken immediately after preparation and after 5, 12, 20, 30, 60, 120, 1000, 1440, and 7320 min; these were used for extraction and quantification of lipolytic breakdown products. Samples (1 mL) of typical emulsions and of emulsions tested for their lipase activity were extracted by mixing these samples with 5 mL of 1:2 (v/v) methanol/chloroform in closed tubes. The tubes were centrifuged (3600*g*, 20 °C, 30 min) and the lower organic layer was collected, evaporated at 40 °C under a flow of nitrogen, and subsequently dissolved in 5 mL of methanol and used for further analysis. This extraction may lead to a small underestimation of the caprylic acid concentration, because a small proportion of this compound remained dissolved in the water phase, at neutral pH.

HPLC analysis was performed with a Spectra Physics P1000 solvent delivery system equipped with an AS3000 autosampler and UV3000 absorbance detector (Thermo Separations Products, Fremont CA). Portions of 20 μL of sample were injected on a Spherisorb 5 ODS-2 column (4.6 i.d. × 250 mm) (Chrompack, Middelburg, The Netherlands) equilibrated with a mixture of 40% (v/v) acetone, 40% (v/v) acetonitrile, 20% (v/v) water, and 0.1% (v/v) formic acid at a flow rate of 1.0 mL/min. Separation was achieved by eluting isocratically for 4 min with the starting eluent, followed by a linear gradient to a mixture of 60% (v/v) acetone and 40% acetonitrile, containing 0.1% (v/v) formic acid. Next, the column was eluted isocratically for 3 min using this mixture. Eluting compounds were detected using a Sedex 55 evaporative light scattering detector (SEDERE, Alfortville, France) set to a temperature of 25 °C and using an air pressure of 2.5 bar. The retention times and peak areas were compared to those of the standard compounds. Standard compounds used were tricaprylin, 1,2-dicaprylin, 1,3-dicaprylin, and 1-monocaprylin (all from Sigma) dissolved in methanol. The presence of 2-monocaprylin was confirmed by connecting the HPLC column to an LCQ ion-trap mass spectrometer (Finnigan Mat, San Jose, CA) equipped with an APCI interface and used in the positive ion mode (vaporizer temperature = 450 °C, sheath gas flow rate = 60, corona discharge current = 5 μA, capillary temperature = 150 °C, capillary voltage = 6 kV, tube lens offset = −10 V).

The caprylic acid concentration in the samples was estimated using an HRGC Mega 5300 GC (Carlo Erba Instruments, Milan, Italy) equipped with a flame ionization detector using helium at a pressure of 30 kPa as a carrier gas. Samples of 10 μL were injected on a J&W Instruments DB-FFAP column of 15 m length, an internal diameter of 0.53 mn, and a film thickness of 1 μm (Alltech, Breda, The Netherlands). All components were eluted using a temperature gradient of 12 K/min from 85 to 210 °C. Caprylic acid (Fluka) was used as a reference.

RESULTS AND DISCUSSION

Aggregation Characteristics of Emulsions Made at pH 7. At pH 7 ($I = 50$ mM) emulsions [10% (v/v) oil] were made with protein dispersions (7.5 mg/mL) of PPI, ASP, PIP, and patatin. Microscopic analysis indicated that emulsions made with patatin showed no droplet aggregation. However, all protein preparations containing protease inhibitors resulted in emulsions that showed extensive droplet aggregation and, therefore, creamed quickly.

The mechanism inducing this droplet aggregation was further investigated. The possible importance of bridging aggregation, resulting from a too low surface excess (Γ), was tested by reducing the volume fraction of oil (φ) in the emulsion from 0.10 to 0.025. Microscopic analysis of the emulsion, however, showed that the extent of droplet aggregation was not reduced. If depletion flocculation would be the mechanism inducing the observed droplet aggregation, then the interactions keeping the droplets together would be quite weak and easily broken in the present case, because the concentration of protein, the only polymer present being able to cause depletion flocculation, was quite low. This was checked by measuring the viscosity of the aggregated emulsions as a function of shear rate. **Figure 1A** shows that at low shear rate the viscosity of the aggregated emulsions made with PPI and PIP was much higher than that of the nonaggregated patatin emulsion. Moreover, the viscosity decreased only gradually with increasing shear rate, which makes the mechanism of depletion flocculation rather unlikely. Next, the presence of homogenization clusters in the aggregated emulsions was investigated. If such clusters were present, the decrease in viscosity as observed in **Figure 1A** should be at least partly irreversible because the breakup of homogenization

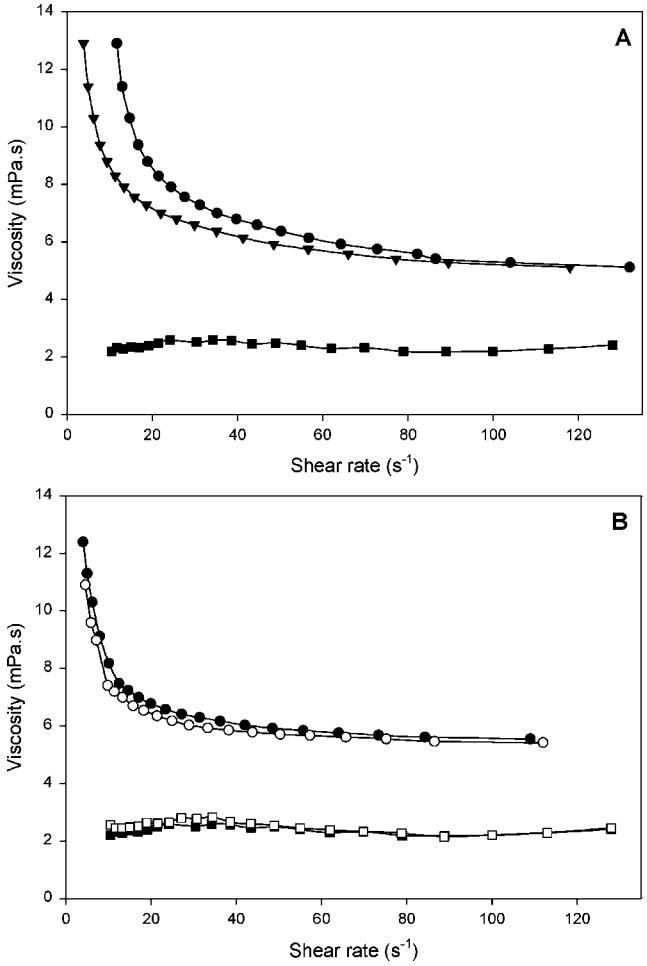

Figure 1. (**A**) Viscosity as a function of shear rate for emulsions made with patatin (■), PPI (●), and PIP (▼) at pH 7 and $I = 50$ mM. (**B**) Viscosity as a function of shear rate for emulsions made with patatin (■, first measurement; □, second measurement) and PPI (●, first measurement; ○, second measurement) at pH 7 and $I = 50$ mM.

clusters is irreversible. **Figure 1B**, however, shows that the viscosity of the emulsions after the first measurement was almost completely recovered. Moreover, microscopic analysis of emulsions that had been passed several times through the homogenizer at a low pressure, which is known to break up homogenization clusters, did not show a reduction in aggregate size. Also, increasing the ionic strength and dilution of the emulsion in 10% (w/v) SDS did not break up the aggregates. Addition of dithiothreitol, on the other hand, caused the aggregates to break up completely into separate droplets. This clearly shows the important involvement of disulfide reshuffling in droplet aggregation in emulsions containing the relatively cysteine-rich protease inhibitors. Using this knowledge, the formation of disulfide bridge-dominated droplet aggregation could be completely prevented by carefully avoiding the inclusion of air during prehomogenization (results not shown).

Other Properties of Potato Protein Stabilized Emulsions. Even when the inclusion of air was prevented, emulsions made with PPI, ASP, and PIP at pH 7 ($I = 50$ mM) showed appreciable droplet aggregation, as can be seen in **Table 1**. However, dilution (1:10) of these emulsions in 0.3% (w/v) SDS before microscopic inspection revealed that droplet aggregation was absent and that therefore covalent interactions were no longer involved in droplet aggregation.

As can be seen in **Table 1**, emulsions made with potato proteins had very small volume-surface average droplet sizes (d_{32}), especially those fractions that contained substantial amounts of patatin (PPI, ASP, and patatin). Emulsions made with PIP at pH 7 ($I = 50$ mM) contained significantly larger droplets than emulsions made with PPI, ASP, and patatin. Surprisingly, emulsions made with PIP and patatin that had been precipitated in the presence of ethanol (PIP-5E, PAT-5E) had a much smaller average droplet size than those made with the untreated proteins. From the emulsions made at pH 7 ($I = 15$ mM) those made with the protease inhibitors in PIP showed a significantly lower surface excess (Γ) of 1.8 mg/m^2 (**Table 1**). The Γ value found for emulsions made with PIP (pH 7; $I = 50$ mM) was found to be unexplainably low and was, therefore, left out of the discussion.

The emulsifying properties of PPI, ASP, PIP, and patatin were also studied at pH 5. Emulsions at pH 5 were made at an ionic strength of 200 mM to keep protein solubility in the fractions containing patatin, at its pI, as high as possible. **Table 1** shows that the average droplet sizes of emulsions made with PPI and ASP were much larger at pH 5 than at pH 7. In emulsions made with patatin the droplet sizes at pH 5 were only somewhat larger than at pH 7. This difference can, however, not be completely attributed to the change in pH, because also the ionic strength was increased from 50 to 200 mM. When prepared at pH 5, the emulsion droplets made with PPI, ASP, and PIP were still aggregated. Droplet aggregation was also observed in emulsions made with patatin at pH 5, a behavior that is often observed for emulsions near the isoelectric pH of the protein used (*28*, *29*).

For the patatin-containing protein fractions (PPI, ASP, and patatin) also a pH 3 → pH 7 treatment was conducted to study at pH 7 the effect of the presence of patatin that had been unfolded at low pH (*30*). Emulsions at pH 3 were made at an ionic strength of ≈15 mM to prevent extensive aggregation and insolubility of pH-unfolded patatin (*19*). In emulsions prepared at pH 3 with PPI, ASP, and PIP droplet aggregation was no longer observed (**Table 1**). All of these preparations contain, in contrast to patatin, large amounts of protease inhibitors with pI values covering the range from pH 5 to 9 (*6*). Because of this wide pI range it is expected that maximum electrostatic repulsion between emulsion droplets covered with these proteins will occur at acidic pH and could, thereby, explain that droplet aggregation in emulsions made with these fractions was absent at pH 3 only. The average droplet size in emulsions made at pH 3 became considerably larger for PPI, PIP, and patatin, but significantly smaller for ASP, compared to the droplets in emulsions prepared at pH 7 ($I = 15$ mM). The surface excess did not change significantly on emulsion droplets prepared at pH 3, compared to the surface excess obtained at pH 7 (**Table 1**).

After the pH 3 → pH 7, treatment the average droplet size in emulsions made with patatin was larger than that at pH 7 without pH 3 → pH 7 treatment. Surprisingly, smaller droplets were obtained with PPI after pH 3 → pH 7 treatment than at pH 7 without pH adjustments. The pH 3 → pH 7 treatment did not result in significant changes in the surface excess on the emulsion droplets (**Table 1**).

Heat treatment (10 min, 80 °C) of the protein fractions before emulsification always gave emulsions with aggregated droplets (**Table 1**). Generally, heat treatment had only a small effect on average droplet size. For all protein fractions heat treatment resulted in emulsion droplets with a much higher surface excess, presumably due to adsorption of protein aggregates (*29*, *31*, *32*).

Apart from the other more protein fraction specific properties, it can be seen in **Table 1** that in general the average droplet

Table 1. Characteristics of Emulsions Made with Various Potato Protein Fractions at Various Conditions[a]

fraction	pH	I (mM)	ΔT	e_0 (mg/mL)	d_{32} (μm)	d_{43} (μm)	c_S (–)	$\Gamma_{protein}$[b] (mg/m^2)	droplet aggregation[c]
PPI	7	15		7.0	0.35	0.83	1.16	2.6	++
	7	50		7.5	0.39	0.91	1.16	2.3	+++
	7	50	yes	11.3	0.49	0.90	0.92	7.8	++++
	5	200		5.2	4.48	5.79	0.54	2.1	++
	3	15		8.1	0.51	0.94	0.92	2.7	–
	3 → 7	50		8.4	0.25	0.81	1.51	2.0	+++++
ASP	7	15		7.5	0.62	1.03	0.81	2.5	+++
	7	50		7.4	0.59	1.08	0.92	2.9	++++
	7	50	yes	11.4	0.52	0.96	0.92	7.7	++
	5	200		3.8	3.01	4.53	0.71	3.8	+
	3	15		7.4	0.43	0.73	0.83	2.6	–
	3 → 7	50		7.7	0.58	0.95	0.79	3.2	++
PIP	7	15		7.7	0.35	1.15	1.51	1.8	++
	7	50		7.5	0.71	2.73	1.68	0.8[d]	+++
	7	50	yes	11.2	0.42	0.99	1.16	6.1	+++
	5	200		7.2	0.73	2.19	1.41	1.7	+++
	3	15		7.8	0.72	1.23	0.84	2.1	–
PIP-5E	7	50		8.9	0.24	0.77	1.51	1.5	++
patatin	7	15		7.5	0.31	0.85	1.31	2.5	–
	7	50		7.8	0.32	0.63	0.99	2.7	–
	7	50	yes	9.8	0.46	1.08	1.16	5.9	±
	5	200		4.4	0.45	0.73	0.79	2.7	+
	3	15		4.8	0.59	0.84	0.64	3.1	–
	3 → 7	50		3.7	0.48	1.84	1.68	2.4	+++
PAT-5E	7	50		11.5	0.20	0.55	1.31	2.6	–

[a] ΔT = heat treatment (80 °C, 10 min); e_0 = protein concentration before emulsification; c_S = relative width of the droplet size distribution; d_{32}= volume-surface average droplet size (μm). [b] Plateau values of surface excess (Γ). [c] ±, ..., +++++ = increasing size of droplet aggregates; – = droplet aggregation not observed. [d] Unexplainable value.

Table 2. Compositions of the Lipid Fractions of Typical Emulsions

	caprylic acid[a] (mM)[b]	monocaprylin (mM)[b]	dicaprylin (mM)[b]	tricaprylin (mM)[b]
before homogenization	14	0	0	200
ASP (heated)	25	22	2	173
PPI (pH 7)	95	19	3	123
PIP-5E (pH 7)	48	33	4	172
PAT-5E (pH 7)	287	40	0	72
patatin (pH 7) just after emulsification	103	21	4	158

[a] Millimoles per liter emulsion. [b] Concentration may be underestimated due to choice of extraction method (see also Materials and Methods).

sizes obtained for emulsions made with potato proteins are rather small for emulsions made with protein as the only surfactant.

Changes in the Oil Composition. Smulders (*29*) found a lowest average droplet size of ≈0.8 μm for emulsions made with various proteins using the same homogenizer and triglyceride as was used in the present study. If we correct this value for the higher homogenization pressure and the lower volume fraction of oil used in this study, we estimate that the lowest average droplet size (d_{32}) attainable with protein as the only surfactant would have been ≈0.6 μm. The average droplet sizes obtained for several of the emulsions made with potato proteins (**Table 1**) were therefore unusually small. Because patatin possesses LAH activity (*7*, *33*), it was hypothesized that patatin might have hydrolyzed tricaprylin and, thereby, have released surface active components such as free caprylic acid and monocaprylin. To test this hypothesis, the oil phase of several emulsions was extracted and analyzed. The estimated concentrations of the different components, based on peak areas, are given in **Table 2**. The data in **Table 2**, of extracts obtained within 1 h after emulsification, show that indeed significant amounts of both caprylic acid and monocaprylin were formed in the various emulsions. The question is now whether we can correlate the observed and expected lipolytic activity in that protein fraction to the droplet sizes (<0.6 μm) in **Table 1**. For the emulsions made with patatin this seems possible. The highest rate of hydrolysis was obtained for PAT-5E, which contains >95% patatin as judged from SDS-PAGE. The activity of patatin in PAT-5E was shown to be considerably higher than that of untreated patatin (*20*), which may indirectly have resulted in the lower average droplet size obtained (**Table 1**). Additionally, the droplets in patatin-stabilised emulsions are larger after heating and at pH 3, that is, conditions that are known to decrease the activity of patatin (*30*, *34*). Also, the lowest rate of hydrolysis, as judged from **Table 2**, is obtained for heated ASP, in which the patatin present is almost completely irreversibly heat denatured. For PPI this relationship is less clear because it does not explain the low average droplet size obtained after the pH 3 → pH 7 treatment, which would reduce the LAH activity of patatin by 35% (*30*). In PIP the presence of patatin could not even be made visible using SDS-PAGE, and emulsions made with PIP should have average droplet sizes higher than 0.6 μm. **Table 2**, however, indicates that traces of patatin, or a similar enzyme activity, must be present, because significant tricaprylin hydrolysis is observed.

From these results it seems, therefore, likely that only a trace amount of active patatin is sufficient to liberate enough surface active compounds from the oil to significantly reduce the surface tension already during emulsification resulting in a decrease in droplet size. To illustrate this, an extract was also made from an emulsion made with patatin at pH 7 directly after emulsification. The composition of this extract showed that during emulsification already 20% of the tricaprylin present had been hydrolyzed and that the ratio monocaprylin/caprylic acid in the

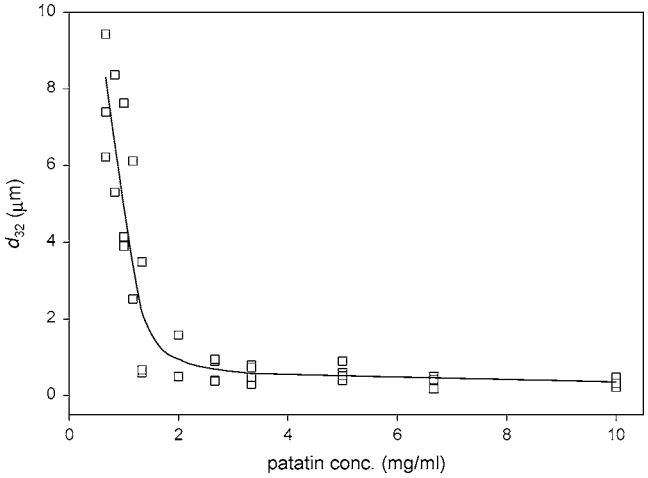

Figure 2. Average droplet diameter (d_{32}) of emulsion made with patatin (pH 7; $I = 50$ mM) as a function of protein concentration (mg/mL).

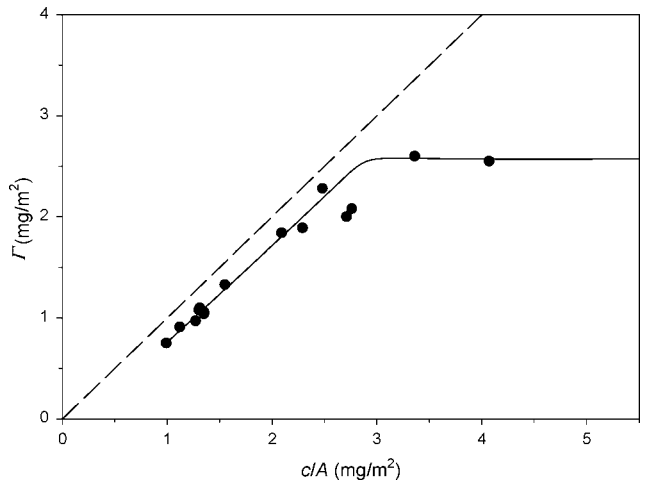

Figure 3. Surface excess (Γ; mg/m^2) of emulsions made with patatin (pH 7; $I = 50$ mM) as a function of c/A (mg/m^2).

emulsion directly after preparation was higher than that after storage for some time (results not shown).

Droplet Size and Surface Excess of Patatin Emulsions. To further characterize the lipolytic properties of patatin in emulsions, its emulsifying properties were studied in more detail. **Figure 2** shows the volume-surface average droplet size of emulsions made with patatin as a function of the patatin concentration. It shows that at low patatin concentration the average size of the droplets formed decreased steeply with increasing patatin concentration. At concentrations higher than ≈3 mg/mL an excess of protein was present in solution and the obtained droplet size continued to decrease more gradually.

In **Figure 3** the surface excess of emulsion droplets made with patatin at pH 7 ($I = 50$ mM) is shown. The surface excess is given as a function of the protein concentration (c) over the specific interfacial area (A) to enable comparison with emulsions made with other proteins and different interfacial areas. At low concentrations the surface excess of emulsion droplets made with patatin increased strongly with increasing concentration, and most of the available patatin was adsorbed at the droplet interface. In **Figure 3** the maximum possible surface excess at any value of c/A is shown as a dashed line. It can be seen that at low concentration the experimental curve for patatin was close

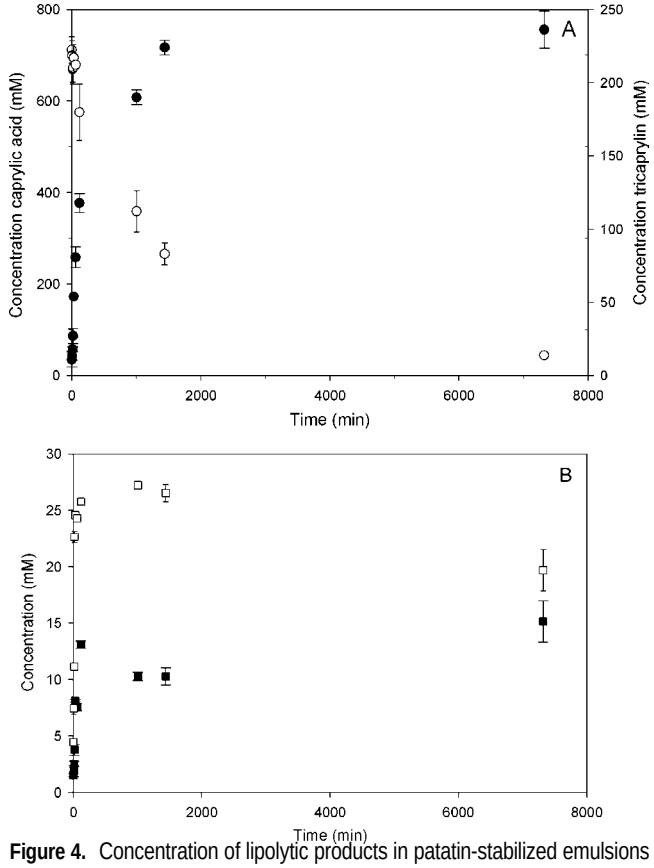

Figure 4. Concentration of lipolytic products in patatin-stabilized emulsions as a function of storage time: (**A**) ○, tricaprylin; ●, caprylic acid; (**B**) ■, monocaprylin; □, dicaprylin.

to this line and remained almost parallel to this line, even at higher concentrations. At still higher concentrations the droplets became saturated with protein and a plateau value for patatin was reached at 2.6 mg/m^2.

It is clear that the small molecule surfactants, which are almost certainly released during emulsification, do not strongly displace the proteins from the droplet surface, since the values obtained for the surface excess of emulsions made with nonheated potato proteins (1.4−3.8 mg/m^2) are comparable to the values normally found for protein-stabilized emulsions (*29*). The absence of protein displacement by moderately high concentrations of oil-soluble small-molecule surfactants, however, seems to be quite general (*17*, *35*).

Lipase Activity in Patatin-Stabilized Emulsions. From the results shown above it has become clear that the activity of patatin on triglycerides has until now been underestimated (*7*−*10*). Therefore, the course of lipolysis as a function of time for a patatin-stabilized emulsion was monitored and is shown in **Figure 4**. **Figure 4A** shows the decrease in tricaprylin concentration and the concomitant increase in the caprylic acid concentration, whereas **Figure 4B** shows the changes in the contentrations of monocaprylin and dicaprylin as a function of time. Whereas both the tricaprylin and the caprylic acid concentrations changed relatively slowly, the concentration of mono- and dicaprylin rose quite rapidly to a maximum, after which the concentration remained more or less constant or decreased a little. The reported high activity of patatin on mono- and diglycerides (*7*−*10*) can be seen in **Figure 4** from the formation of caprylic acid as the main product and the presence of only relatively low concentrations of mono- and diglycerides during lipolysis.

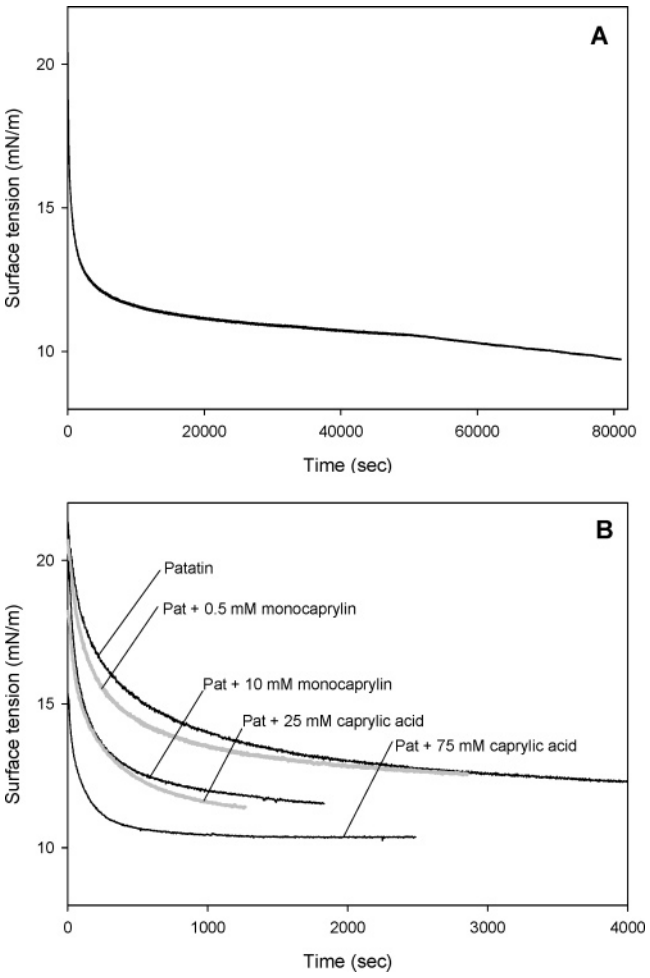

Figure 5. Interfacial tension at tricaprylin/water interface as a function of time: (**A**) oil droplet in patatin solution (4 mg/mL; pH 7; $I = 15$ mM); (**B**) oil droplet in patatin solution (4 mg/mL; pH 7; $I = 15$ mM) with oil containing no additives, 0.5 or 10 mM monocaprylin, or 25 or 75 mM caprylic acid.

During the first 120 min patatin released ≈373 mM caprylic acid (**Figure 4A**), resulting in a rate of 3.11 mM/min. Using a d_{32} of 0.41μm and a Γ of 2.6 mg/m^2, it can be calculated that the lipase activity of patatin on this substrate was ≈820 units/g (μmol g^{-1} min^{-1}), which corresponds to a turnover number of 0.55 s^{-1}. The lipolysis rate generally is proportional to the amount of interfacial area formed, when the amount of enzyme is not limiting (*36*). The lipase activity of patatin is relatively low because turnover numbers of up to 3000 s^{-1} have been reported, and most commercial lipases have activities far above 10000 units/g (*37*, *38*). However, compared to other plant lipases (*39*) the activity is considerable.

Effect of Lipolytic Breakdown Products on Interfacial Tension and Droplet Size. The presence of monoglycerides and fatty acids in addition to proteins is expected to decrease the average droplet size of emulsions significantly, as do other oil-soluble small-molecule emulsifiers (*35*, *40*). To investigate what the effect of monocaprylin and caprylic acid would be on the interfacial tension during emulsification, representative quantities (see last row of **Table 2**) of both compounds were added to a patatin solution, and the interfacial tension was subsequently monitored as a function of time using a droplet tensiometer (**Figure 5**). **Figure 5A** shows the interfacial tension of a tricaprylin oil droplet in a patatin solution as a function of time. The interfacial tension starts at a value of 22 mN/m, a value also found by Smulders (*29*). The initial decrease in interfacial tension would be mainly due to the adsorption of patatin to the oil/water interface, whereas with the advancing of time also the released lipolytic products will act on the interfacial tension. The latter effect can also be seen from the continuing decrease of the interfacial tension even after 24 h. In an emulsion the effect of lipolysis would be more apparent as the ratio of interfacial area to volume would be higher, thus favoring the lipolytic reaction and the accumulation of its products at the interface.

Table 3. Volume/Surface Average Droplet Sizes of Emulsions Made with Inactivated Patatin at Various Monocaprylin and Caprylic Acid Concentrations

caprylic acid (mM)[a]	monocaprylin (mM)[a]	d_{32} (μm)
0	0	2.10
0	5	1.26
0	25	0.49
25	0	0.57
100	0	0.32
50	12.5	0.37

[a] Millimoles per liter of emulsion.

It can be seen in **Figure 5B** that already small quantities of monocaprylin cause the decrease in interfacial tension to become much faster. The relatively large amount of caprylic acid released by the patatin results in an even stronger decrease in the interfacial tension. It can be anticipated that both compounds may have a severe effect on the emulsification process and the final droplet size of patatin containing tricaprylin emulsions. To test this effect emulsions were made from patatin solutions of which the LAH activity had been irreversibly inhibited by methyl arachidonyl fluorophosphonate addition and various amounts of monocaprylin and caprylic acid had been solubilized in the oil phase. The average droplet sizes of these emulsions are presented in **Table 3**. Although, due to the use of a different method to estimate average droplet sizes, a direct comparison between **Table 3** and the other data in this study is not possible, it can be seen that the presence of monocaprylin or caprylic acid had a strong effect on d_{32} (**Table 3**). Similar to the effects on surface tension (**Figure 5B**), caprylic acid, although studied in a somewhat higher concentration range, also had a strong lowering effect on the average droplet size. When caprylic acid and monocaprylin are put together at concentrations that can be regarded as low for patatin emulsion (see **Table 2**), there seems to be a synergistic effect on the average droplet size of the emulsion obtained.

This study shows that trace amounts of patatin suffice to cause extensive lipolysis. In the presence of active patatin the average droplet size of the potato protein stabilized emulsion is dominated by the release of fatty acids and monoglycerides, whereas the presence of protease inhibitors seems to determine the droplet aggregation behavior and, thereby, the stability against creaming, of potato protein stabilized emulsions.

The application of potato protein in emulsions would, as can be concluded from this study, preferably be done at acidic pH. Heat denaturation of the patatin present, which would also diminish the protease inhibitor activities (*19*, *20*), could be done at low pH as suggested by Ralet and Guéguen (*14*) in order to prevent excessive protein aggregation. Also, the use of medium- and long-chain triglycerides should diminish the lipolytic activity of patatin in large part (*7*−*10*).

ABBREVIATIONS USED

A, specific interfacial area (m^2/m^3); ASP, ammonium sulfate precipitate; *c*, protein concentration; c_S, relative width of the droplet size distribution; cv., cultivar; d_{32}, volume-surface average droplet size (μm); E_{SD}, surface dilational modulus $\equiv$ dγ/d ln *A*; *I*, ionic strength; LAH, lipid acyl hydrolase; n_D, refractive index; p_L, Laplace pressure $= 2\gamma/R$ (Pa); PAT-5E, patatin resolubilized at pH 7 after precipitation at pH 5 in the presence of 20% (v/v) ethanol; PJ, potato juice; PIP, protease inhibitor pool; PIP-5E, PIP after precipitation at pH 5 in the presence of 20% (v/v) ethanol; PPI or PPI (15% EtOH), potato protein isolate prepared by precipitation at pH 5 in the presence of 15% (v/v) ethanol; PPI (20% EtOH), potato protein isolate prepared by precipitation at pH 5 in the presence of 20% (v/v) ethanol; γ, interfacial tension (N/m); Γ, surface excess (mg/m^2); η_{SD}, surface dilational viscosity [$= \Delta\gamma/(\text{d ln } A/\text{d}t)$]; ρ, density of liquid phase (kg/m^3).

ACKNOWLEDGMENT

Potatoes were kindly provided by AVEBE B.A. (Foxhol, The Netherlands).

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Received for review May 7, 2006. Revised manuscript received June 16, 2006. Accepted June 20, 2006. This research was supported by the Ministry of Economic Affairs through the program IOP-Industrial Proteins and by AVEBE B.A.

JF061278Z