Potato Proteins: Genetic and Physiological Changes, Evaluated by One- and Two-dimensional PAA-Gel-techniques

H. Stegemann, H. Francksen, and V. Macko *

Institut für Biochemie, Biologische Bundesanstalt, Braunschweig

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The proteins of mature potato tubers can be used for variety identification. Their pattern after electrophoresis or focusing are genetically determined. Various growth regulators applied during the season had no influence. Molecular weights of the protein subunits are uniform and decrease with age of the tuber, often due to depolymerization. Some subunits are linked by S–S-bridges. A proteid characteristic for immature tubers has been discovered. Part of the diversity in charge distribution is based on different degree of amidation; treatment at pH 10 changes the variety-dependent pattern to a picture similar in all varieties. The latter is also true for proteins from immature tubers. Focusing followed by SDS-incubation (with and without ME) and SDS-gelelectrophoresis revealed that the main proteins which were different in charge had common MW and may derive from a parent protein.

The tuber proteins and especially some of the peroxidases are quite resistant to SDS-attack. Only prolonged incubation (50 °C, 40 hours) with SDS brings about digestibility. A borate buffer, pH 7.9 has been used, making possible the electrophoresis of undiluted, unfractionated sap or other crude mixtures of proteins. Some new versions of two-dimensional techniques with PAA as a carrier are applied in order to differentiate between charge, shift of charge, and MW.

Elucidation of the structure and function of plant proteins must ultimately depend on characterization of individual proteins and their change due to physiological and pathological conditions. This will be an unconditional prerequisite if patterns of proteins or isoenzymes are to serve to chemotaxonomical ends and provide for genetic markers. So far little is known about the diversity of proteins in the potato tuber (*Solanum tuberosum*), their alteration among and relationship to other macromolecules caused by external or genetic factors. We will deal with soluble proteins, stainable by nonspecific dyes to obtain information on storage proteins beyond the specific case. Advanced separation methods are applied which minimize the steps of prepurification and are adaptable generally to plant and animal proteins.

Separation of tuber proteins has been achieved by ion exchange chromatography, which gives largely the same pattern as paper electrophoresis [1], or by polyacrylamide electrophoresis, which results in better resolution [2, 3]. It was discovered, that the distribution is genetically determined and therefore a tool for the differentiation of varieties [4–6]. This was summarized in [7]. Recently a critical remark was published [8]. Furthermore, identification of varieties by electrofocusing and mapping has been achieved [9]. There is no information on the extent of the complexity or distribution of the proteins in respect to tissue topography and molecular weights nor is it clear whether the numerous proteins are charge isomers of a few parent proteins or have different primary structures. This work will elucidate some of these properties.

Materials

Potatoes were supplied by the Bundessortenamt, D 3161 Rethmar (Frau Dr. E. Höppner), or grown in our fields. Growth regulator treated potato plants were grown in the Forschungsanstalt für Landwirtschaft (Braunschweig-Völkenrode). We are indebted to Dr. Chr. Pätzold for samples. Storage was at 6–10 °C, if not otherwise stated. For sap preparation tubers of medium size were frozen at −18 °C, thawed at 6 °C over night and peeled. The sap was forced through a dense filter-cloth (perlon

Abbreviations: MW, Molecular weight; SDS, Na-dodecylsulfate; Tris, Tris-(hydroxymethyl)-aminomethane; PAA, polyacrylamide with methylene-bis-acrylamide; ME, mercaptoethanol.

* Present addres: Boyce Thompson Institute for Plant Research, 1086 North Broadway, Yonkers, New York 10701, USA.

Requests for reprints should be sent to Prof. Dr. H. Stegemann, Institut für Biochemie, Biologische Bundesanstalt, *D-3300 Braunschweig*, Messeweg 11/12.

4438 fix., Weberei Weidmann Co., D 7334 Süssen) or through 2 crosswise laid sheets of PVC-paper (Rhovylpapier 1001, Schleicher & Schüll, D 3354 Dassel) by a hydraulic press either hand operated (type SPH 2,5, Seifert KG, D 755 Rastatt) or motor driven (type HAFICO-Presse, H. Fischer Co., D 4041 Norf bei Neuß). For smaller samples up to 1 cm^3, a press made from plastic (see below) was used. Sap used in SDS-electrophoresis had to be dialyzed to avoid precipitation of potassium dodecylsulfate.

For topographic analysis of a tuber the tissue was taken from peeled, large-size potatoes: a. 2 – 3 mm of the cortex zone, b. 3 – 5 mm of a layer including the concentric bicollateral ring of vascular bundles, c. a layer of 5 mm of pith tissue and d. the parenchymatous center (medulla) with a diameter of about 1 cm, essentially free of vascular bundles. From the bud and stem end a cone with a base of roughly 2 cm^2 was cut off. If not otherwise mentioned the tuber was kept frozen, thawed immediately before use, the juice was extracted by a nutcrackerlike press (mainly made from high-density-polypropylene and polyamide to avoid contact with metals, Labor-Müller, D 351 Hann. Münden) and handled as usual.

To 100 ml of the ice cold sap, 2 ml aqueous solution of 100 mg Na_2SO_3 and 75 mg $Na_2S_2O_5$ (freshly dissolved) was added and the mixture cleared ($15000 \times g$) in a refrigerated centrifuge. In special cases, no sulfite was added. The sample was split into 1 ml-portions which were quickly frozen. For further details see l. c. [10]. Evaluation of various procedures for preparing juice see l. c. [3].

Serum of trouts was a gift from Dr. A. Keese, Institut für Tierzucht, University of Göttingen, rabbit serum was given by Frau Dr. R. Koenig, Institut für Virusserologie, BBA, Braunschweig, human serum was supplied as Seretin and Standard-Human-Serum from Behring-Werke, D 3550 Marburg. *Peroxidase "c"* from horse-radish was a gift from Dr. L. M. Shannon, Riverside (USA), all other enzymes were of the purest grade available.

Chemicals were of pure grade, since the more expensive analytical grade gave no better performance. Acrylamide (tech. grade) was ordered in bulk (Shell-Chemie, Frankfurt) and used for all electrophoretic procedures in analytical runs in 3 mm slabs. For preparative runs and as a carrier in electrofocusing acrylamide and methylene-bis-acrylamide were recrystallized from chloroform and acetone respectively. Cyanogum 41 pract. was supplied by Serva, D 69 Heidelberg, Ampholine by LKB-Producers, Bromma, Sweden. Marker dyes in gel electrophoresis: Evans blue, Cibacronbrillantgelb 3G-E, Reactonviolett S-RL, Drimarenmarineblau X-RBL, Reactonmarineblau S-RBL, Cibacronblau B-E, Amidoschwarz 10 B, Tropaeolin 00, Cibacronschwarz, Bromphenol blue, dissolved in traces of methanol first, then buffer was added. They were gifts from Ciba, Geigy, and Sandoz. Marker dyes which could be applied together with proteins: Bromphenol blue, Amidoschwarz and Tropaeolin 00. Supranolcyanin 6B Bayer was almost exclusively used for protein staining *.

Buffers: Tris (0.125 M)/borate-buffer pH 8.9 contained 15.12 g Tris and 1.15 g boric acid in 1 l [3], Tris (0.03 M)/borate-buffer pH 7.9 contained 3.65 g and 4.40 g in 1 l respectively. They were used for standard-electrophoresis, the buffer pH 7.9 was also used for dialysis. Tris (0.04 M)/borate/EDTA-Na-buffer pH 8.2 (4.85 g, 3.0 g and 0.6 g in 1 l) was used occasionally for dialysis. For determination of molecular weights by gel electrophoresis after loading proteins with SDS according to [11] a more diluted 0.025 M phosphate buffer pH 7.1 was used: 12.5 g $Na_2HPO_4 \cdot 12\,H_2O$ and 2.1 g $NaH_2PO_4 \cdot H_2O$ were dissolved in 2 l water, 2 g SDS were added.

Methods

Dialysis and concentration were performed in collodion bags (Membranfilter-Gesellschaft, D 34 Göttingen, or Schleicher & Schüll, D 3354 Dassel) against the above mentioned buffers pH 7.9 or pH 8.2 with 0.05% ME under reduced pressure (500 mm Hg) in a corresponding flask. Before use the collodion bags were checked for retention properties [12] with carbamylated chymotrypsinogen A [13]. For analytical gel electrophoresis solutions could be concentrated in a visking tubing by surrounding it with Carbowax 20 000 (polyethyleneglycol, Serva, D 69 Heidelberg). In this case impurities of low molecular weight Carbowax passing through the tubing did not interfere. Lyophilization should be avoided.

Fast estimation of protein concentrations in numerous fractions was done by spotting 2 cm apart 5 μl samples with 0.1 – 5 μg protein on paper (Schleicher & Schüll, D 3354 Dassel, type No. 2043 a). The strips were dried, soaked for 2 min in 20% sulfosalicylic acid, stained with Supranolcyanin 6B Bayer or Coomassie Blue (0.25% in water) for 5 min [14, 15] and destained with water/methanol/acetic acid (28/12/2, v/v/v) three times for 3 min. Protein concentrations could be estimated by comparison of spots containing known amounts.

* We are indebted to the manufacturers especially to Bayer (Leverkusen) giving us different lots and analytical datas of the dye.

Electrofocusing was carried out either in tubes (100 mm long, 5 mm i.d.) according to [16] and the adjustments of [9] in 6% acrylamide and 0.16% bisacrylamide (polymerized by 20 mg ammonium persulfate and 0.065 ml tetramethylethylenediamine per 100 ml) with 1% Ampholine for 4 hours at 200 – 250 V in an apparatus with 2 rows of 8 tubes [17] or without a carrier in a polyethylene tubing [18]. Focusing was also performed in rectangular plates at 10 °C photopolymerized in the flat gel apparatus [12]. All parts are available from Labor-Müller, D 351 Mann. Münden.

2 slightly greased spacers (170 × 14 × 2 mm) are placed at the long sides of a clean glass plate (17 × 15 × 0.3 cm) put into the lower part of the apparatus in the horizontal position. A thin line of grease was applied beneath the glass from one wall to the other in order to have a tight barrier between the electrodes. 1 plexiglass-plate (125 × 17 × 5 mm) is put at the back, another one (125 × 23 × 5 mm) in front of the smaller sides of the glass-plate. Then the spacers were covered with a plate (bearing 8 slot formers 2 × 2 × 10 mm) and the upper part of the apparatus. 75 ml of the monomer mixture containing 3.4 ml Ampholine, 150 µg riboflavin and 10 mg glycine was poured in avoiding air bubbles. After gelling the covers and the 2 pieces at the smaller sides of the glass-plate were carefully removed, the empty spaces filled with 0.1% phosphoric acid for the anode, with 0.1% ethanolamine (distilled) for the cathode and 2 graphite-electrodes were inserted. 200 V were applied 4 times for 15 min, the solution in the reservoirs being replaced each time. Then water from the slots was removed by filterpaper and 30 µl of the samples applied. If their protein concentration is low, a paper S + S 2043 a (10 × 10 mm) is soaked with the sample too and applied on top of the filled slots. If the salt concentration is high dialyze against glycine (1%) or Ampholine. After 30 min focusing at 500 V (gel not covered during this time) the upper part of the apparatus is used for covering and additional cooling. Focusing is continued for 1 hour at 700 V and 30 min at 1000 V or for a shorter time at a higher voltage. Staining was done according to [20]. The protein fixation was done immediately after switching off the current and transferring the gel slab into a stainless tray.

Polyacrylamide electrophoresis with or without continuous elution or the mapping was accomplished in a flat gel apparatus [12, 21] adaptable for analytical or preparative runs by different spacers *.

* Apparatus, adapters and additional parts available from Labor-Müller, D-351 Hann. Münden.

Analytical electrophoresis was done in slabs of 3 mm thickness, 8 samples per run, in Tris/borate-buffers pH 8.9 or 7.9. The gel was made from 7.5 g Cyanogum-41 in 150 ml buffer, adding 50 mg Na-sulfite, 0.35 ml dimethylaminopropionitrile and 2.8 ml of 2% amonium persulfate solution. The cooling was set first to 15 °C, decreasing to 2 °C. 20 – 50 µg of protein in 10 – 40 µl (maximum 100 µl) were applied into one slot and separation took place at 500 V (not more than 50 W) for 110 min **. One corner of the slab was cut off for identification and the staining performed with 0.025% Supranolcyanin 6 B Bayer in 200 ml of a mixture trichloroacetic acid, water, methanol, acetic acid (6/80/20/7 = w/v/v/v) for 3 hours. For staining over night the dye concentration was lowered to 0.01%. For gels containing SDS staining time was increased to at least 9 hours. Destaining was done in water, methanol, acetic acid (28/12/2). Preparative runs were done in gels 16 mm thick, cast horizontally aided by a newly designed surface former. It is similar to a 16 mm block with handle, placed 12 mm deep between the cooling plates and removed after gelling. This saved an extra gel base and the surface preparation in contrast to a method described previously [21]. The idea came from Dr. Wolf, Göttingen. Gel sheets of 2 mm were sliced off for specific staining, the rest of the slab was worked up by cutting out the spots under investigation.

Two dimensional separations were performed in slabs 3 mm thick and rods 5 mm in diameter. Five versions have been done: Focusing/electrophoresis (mapping) according to [9]; focusing followed by incubation with SDS/electrophoresis in SDS-gel; electrophoresis/focusing; electrophoresis followed by incubation with SDS/electrophoresis in SDS-gel; as well as electrophoresis/electrophoresis at two different pH-values or/and gel concentrations. While this manuscript was in preparation the combination electrophoresis/SDS-electrophoresis was published for ribosomal proteins [22].

Electrofocusing was done in tubes (see section "Electrofocusing") followed by incubation (60 min) of the separated proteins in the PAA-rod with 50 ml 0.5% SDS per rod with or without 0.5% ME in 0.025 M phosphate buffer pH 7.1. Focusing time ranged from 3 hours for Ampholines pH 5 – 7 to 5 hours for Ampholines pH 4 – 6. The following electrophoresis was performed in slabs of 3 mm using the special slotformer for the rod and the unfocused sample [12] in a 0.025 M phosphate buffer

** For more detailed description see: Direction for use of the apparatus for gel electrophoresis and focusing, 8 p., 1973. Will be sent on request.

pH 7.1 with 0.1% SDS for 3 hours at 200 V and approx. 120 mA. If the proteins were to be reduced after focusing, a preincubation of the PAA-rod for 10 min with 1% ME in buffer pH 7.1 was advisable, the solution was discarded and SDS-incubation was performed for 50 min in a buffer containing 0.5% mercaptoethanol and 0.5% SDS.

Electrophoresis followed by focusing was done correspondingly using single slot formers with a basis 3×10 mm for the electrophoretic run. Before performing focusing in slabs a strip 7 mm wide and about 10 cm long (cut out of the slab after electrophoresis) or a gel rod was washed with 50 ml water for 20 min, placed rectangular to the current into the apparatus [12] horizontal in position and polymerized into the gel containing 1% Ampholine. ME was added if needed, but care was taken not to inhibit polymerization.

Electrophoresis followed by gel electrophoresis with SDS, in a different buffer or into a gel of different concentration was performed in the manner mentioned above with minor modifications. In preparative work a gel thickness of 16 mm was applied.

All staining procedures with Supranolcyanin and destaining were done according to the section "Electrophoresis" and not as in section "Electrofocusing" [20].

Results

New buffer for general application, marker dyes

A new buffer pH 7.9 of high boric acid concentration gives better separation in many instances compared with the most commonly used Tris/borate- or Tris/borate/EDTA-buffer of pH 8.9 and 8.2 respectively [3]. To verify the broad qualification human (Seretin), rabbit and trout serum and the proteins of four potato varieties have been tested and compared (Fig. 1 *). The direct application of the unfractionated, salt containing sample was possible. On the other hand more than 15% of saccharose for making the sample denser should be avoided. The buffer of pH 7.9 does not tolerate as much sugar as the buffer of pH 8.9. Longer storage of polyacrylamide gels in the buffer pH 7.9 before electrophoresis did not influence the protein pattern, whereas a gel kept at pH 8.9 for a week gave quite different migration rates of potato proteins and the rise of a few very distinct (artificial?) zones. Practicable marker dyes for electrophoresis with good distribution over the whole gel have been used for locating proteins. 7 dyes (out of 60 tested) were used and are mentioned in the section "Materials" in the order of increasing mobility at pH 8.9. However, there is more or less interaction with the proteins except with Tropaeolin 00, Bromphenol blue and Amidoschwarz. Therefore the interfering marker dyes were applied into a separate trough.

Anodic mobility is comparable only if heights and trends of voltage and current under otherwise identical conditions are the same. The difficulties of comparing different runs in tubes were too great, therefore slab techniques were applied whenever it was possible. Reproducibility obtained can be checked in Fig. 7 and Fig. 8.

Influence of pH and temperature. Treatment at pH 10

After conventional preparative electrophoresis [21] at pH 8.9 eluted bands of tuber proteins showed additional, faster running bands if re-electrophoresis was done in the same buffer. This was essentially prevented by keeping the fractions at 0 °C throughout, use of buffers of pH 7.9 or neutralizing the eluat with CO_2, and adding ME, especially during concentration in collodion bags. To check the heterogeneity of a single band the extracted proteins, homogeneous in gel electrophoresis at pH 7.9 and at pH 8.9, were electrofocused in Ampholine pH 5 – 7. The number of zones or the number of spots after mapping dependend on storage time and temperature of the sample, the satellite spots being more acidic mostly.

To strengthen this observation, tuber proteins were dissolved in 0.04 M Tris/borate/EDTA-buffer pH 8.2, brought to pH 10 by adding 0.5 M Na-carbonate buffer and incubated up to 20 hours at 50 °C. This treatment brings about a faster migration in gel electrophoresis. This was more pronounced after longer incubation and is well demonstrated, if fractions isolated by preparative electrophoresis are used (Fig. 2). The stronger protein zones of low mobility changed completely to faster ones. The group close to the anode disappeared as well as the characteristic pattern of the variety and yielded a diffuse background staining near the starting line after electrophoresis (Fig. 3).

If the pH 10-treated proteins were subjected to MW determination most of the proteins in the region 100 000 – 25 000 had disappeared, especially the proteins with MW around 90 000 and 37 000

* Figs 1 – 17 see Tables on pages 728 a – d.

(Fig. 4). Those subunits of less than 20 000 daltons still have the same position. A general decrease of stainable proteins has been observed.

ASN, GLN and GLN-GLN were subjected to similar treatment. Deamidation was traced after high voltage electrophoresis on thin layers [23]. Glutamic acid appears after 4 hours at 50 °C and GLN is completely converted after 24 hours. For detection by ninhydrin 3 N HCl (40 hours, 50 °C) was used to hydrolyze the pyrrolidone carboxylic acid formed to glutamic acid. The intensity of spots in Fig. 5 demonstrates the rate of deamidation. Asparagine was not hydrolyzed under these conditions.

Ageing: Protein concentration, electrophoretic pattern, molecular weights

Tubers of the early varieties Lori and Sieglinde and the late varieties Maritta and Voran and some others were harvested in monthly intervals from July until October and further samples were taken between November and June. The tubers have been analyzed for proteins and amylases, phosphorylases (Dr. Shivaram), peroxidases and esterases (Dr. Loeschcke). Only proteins will be considered from small and large, young and old tubers and only a few examples of roughly 4000 are given here. The protein composition of cuts from different tissue will be described later.

From "tubers" (almost stolons) of 5 mm diameter very little protein could be extracted. A sharp rise in the extractable proteins was found for bigger tubers keeping a high level (1 – 2%) until they sprouted, decreasing to zero level in the mother tuber of a new potato plant analyzed in August. Tubers at least 4 cm thick harvested in August have the same protein spectra compared with tubers harvested in September, October and November, and differed only in the intensity of very few zones for the August-tuber, whereas the tubers gathered in July show a quite different electropherogramm (Table I). The spectra were constant between September and February and shifted again when sprouting began. An example for Lori and Maritta is given in Fig. 6. Also the proteins extracted from tubes varying in size (2 – 7 cm) were compared, the separation of proteins were done at pH 7.9 and 8.9. The spectra were the same for larger and smaller tubers if the date of harvest was late in contrast to some aberration for very small tubers harvested in July and August. Fig. 7 shows proteins of mature tubers 2 – 7 cm in diameter and serves as check for the scope of identity of the electrophoretic pattern.

In contrast to the typical electrophoretic pattern of mature tubers the protein spectra of immature tubers harvested in July were far less characteristic of the variety (Fig. 8). The predominant zones stained with protein dyes were alike and their migra-

Table I. Shift of protein spectra during growth, storage and sprouting of large and small (2 cm ϕ) potato tubers. Reference: Tuber proteins in November. Harvest in October.

	Maritta		Voran		Lori		Sieglinde	
	large	small	large	small	large	small	large	small
1972								
July	△△△	△△△	△△	△△△	△△	△△△	△△△	△△△
August	△	△	○	△	(△)	△	△	△
September	○	○	○	○	○	△	○	○
October	○	○	○	○	○	○	○	○
November	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
1973								
January	○	–	○	–	○	–	○	–
February	(△)*	–	○*	–	△**	–	△**	–
March	△**	–	△*	–	△**	–	△***	–
April	△△***	–	△△**–		△***	–	△***	–

△△△, Difference great to reference; △△, difference to reference; △, difference noticeable; (△), difference barely noticeable; ○, no difference; *, intensity of sprouting.

tion rates were the same at pH 8.9 and pH 7.9. This causes an arbitrary relationship to the pH-dependent mobilities of the variety-specific proteins. The atypical more uniform pattern disappears in August except for some weak but sharp zones (sometimes jagged) which may merge with or separate from neighbouring zones if electrophoretic conditions change very little.

The size distribution of proteins in mature tubers was almost independent of the variety, if we determined the subunits after treatment with SDS at pH 7 and 37 °C [11, 24]. In the dormant tuber there were only 3 principal proteins of the MW 16800, 18000 and 19500, when no ME was added, followed by a group of proteins of MW 34500, 36500 and 39500 about a tenth in concentration, which is almost exactly twice the MW of the former group. If S – S-bridges were reduced, the faster of the main subunits split into at least 2 even faster migrating zones of MW 13800 and 10200. Lower MW cannot be estimated by this method. A comparison of different incubation time at pH 7 with and without ME is shown in Fig. 9. Proteins of the MW-group 36000 disappeared in favour of a few bands of higher MW when the sap was incubated without ME at pH 9 instead of pH 7. The 3 main bands after focusing had the same MW-subunits and the 2 smaller ones derived from one of the main zones after ME-treatment had a similar size distribution too as revealed by twodimensional techniques (Fig. 10). The patterns were compared by the mapping method without SDS (Fig. 11). If the proteins were treated with iodoacetamide, one zone of a MW of 15200 appeared at the expense of the zone representing a protein subunit of the MW 13800 (Fig. 12).

The molecular size of the subunits of stainable tuber proteins shifted from larger weights in young tubers to smaller units in sprouting ones. The size distribution can be reversed. If old tubers were taken and the tissue was forced to regenerate by multiple cutting into slices of about 1 mm the pattern of a very young tuber was regenerated to the extent of new cell layers formed (Fig. 13). In tubers of about 2 cm in diameter the MW of the proteins were not much influenced by reduction of disulfide bridges (*e. g.* variety Lerche). In varieties predestinated to premature tuber formation (*e. g.* variety Merkur) the small tubers contained an excess of the dimers mentioned in the next section.

The error of the MW-determination was less than 10%. The length of incubation time with SDS was 3 hours at 37 °C and increased if needed up to 40 hours or 100 °C. The spectra of the storage proteins did not alter with longer incubation time in contrast to some peroxidases of the tuber. Runs in 5, 7 and 10% Cyanogum revealed, that here the migration distance in relation to the marker proteins varies with the gel concentration, if activity-staining is used. The completely unfolded part had no activity as expected and behaves probably normal in SDS-gels. This was deduced from and checked with purified horse-radish peroxidase c, stained with H_2O_2/dianisidine and supranolcyanin respectively [25]. The unfolding of potato peroxidases with increasing incubation time is demonstrated in Fig. 14. The horse-radish peroxidase c, pure enough to be stained with an unspecific dye, showed a strong influence of the gel porosity not fitting with the migration rate of the marker proteins. Only if the incubation time and temperature was increased to 4 hours at 50 °C, its mobility followed the rules for unfolded proteins in gels of different porosity [25].

Action of proteinases

The storage proteins and the peroxidases were quite resistant to proteolytic attack. Trypsin, chymotrypsin, papain, leucineaminopeptidase and pronase-P had little effect, even at concentrations up to 10% of the tuber proteins. Nagarse (Serva) at the same or a 5 fold concentration digested the storage proteins stainable with supranolcyanin, whereas the peroxidases of potatoes or horse-radish were not affected. The activity stain reveals no loss (Fig. 15). 5 – 100 μl sap were separated by gel electrophoresis in polyacrylamide, which had included 0.1% gelatine [15]. The slab was incubated for 20 hours at 40 °C and pH 8.9 to see proteolytic action but no "holes" were detected after staining. The same was true for a sap five fold concentrated, corresponding to 5 mg protein. Treatment with 1% SDS under standard conditions [11, 26, 27] for 3 hours at 37 °C destroyed most of enzymatic activities and the proteins became digestable by proteinases.

To check self digestion, the centrifuged sap was incubated at 40 °C either as such or concentrated by carbowax 20000 with or without mercaptoethanol. Electrophoretic spectra of the treated and untreated samples were the same. Tuber proteins pretreated with ME and incubated at pH 10 coagulate

on the surface of the polyacrylamide during electrophoresis, regardless whether persulfate had been removed by preelectrophoresis or not.

Protein distribution relative to sample sites

The peel itself has some proteins not matching completely with the normal pattern and was therefore always discarded. The bud end and the stem end as well as the other cuts from the peripheric to the center region of a peeled potato displayed similar protein spectra when checked with nonspecific stains only in contrast to findings with enzymes. The protein concentration based on sap volume increases approaching the center and was most apparent for the slowest migrating proteins (Fig. 16). There was a shift of the relative protein concentration from the bud to the stem between May and July, mainly between June and July for potatoes in storage, one month earlier for planted potatoes. At the end of the growing season the bud region was richer in all the proteins regardless of the variety and the shift late in spring was also of a general nature, the date being somewhat influenced by the type of variety (early or late sprouting).

Influence of growth regulators

There was no influence on the protein distribution, if the potatoes were grown in the field or in pot trials (Mitscherlich) with our without treatment by 2,4-dichlorophenoxyacetic acid (Hedonal 1.5 l, 1%/ha), maleic hydrazide (MH 30, 600 l, 0,3%/ha), Diquat (Reglone, 5 l/ha), 2,4-D-isopropylic ester with inorganic ions (NCL 6 + 6 kg/ha and the same 12 + 12 kg/ha) with an interval of 14 days [28]. In general the protein concentration of the field grown potatoes was higher, but this was not tested in several seasons (Fig. 17). It should be mentioned, that fertilization was the same. More nitrogen did not change the pattern but brought about an increase of all zones.

Discussion

Many proteins of seeds and storage tissues of plants are interesting in so far as their quantitative relationship is dependent on the genetic background. Neither their function nor metabolism are understood nor their role as precursors. The protein pattern of mature potatoes is a tool for a fast identification of varieties, it is independent upon soil and climate [7]. The pattern is influenced by the age of the tuber, however, precise datas of the shift were not known. For variety-evaluation of ripe potatoes the elucidation of the physiological change may not be needed, but if spectra of soluble proteins are the basis for a general catalogue of varieties [29] and those proteins or isoenzymes will serve as genetic markers, the rules of interdependences should be established.

Plant proteins – in general – can not be worked up as easy as animal or bacterial proteins. Their globular nature restricts solubility, the abundant oxidases in plant tissues especially phenoloxidases cause tanning. Therefore fast separation procedures without prepurification are needed. Since PAA-gels can tolerate concentration of salts ordinarily present in tissue, techniques with PAA as a carrier were adopted for separating proteins from crude saps after centrifugation. Even very simple procedures such as dialysis have been abandoned as far as possible to speed up the process and to diminish the risk of losses and conversions. The accelleration is most important if a large number of samples must be handled for genetic analysis. So, we dealt with soluble proteins obtained after freezing and squeezing the thawed tissue. Freezing was introduced [10] because the protein spectra showed sharp lines compared to more diffuse but identical bands caused by cell disruption by other methods.

The resistance to self digestion of proteins in sap from potato tubers is partly due to the abundance of proteinase inhibitors, which can even take care of an excess of added proteinases. Potato proteins extracted from a gel or eluted after preparative gel electrophoresis are also not attacked by trypsin or chymotrypsin unless they are denatured by heat. For a fingerprint this has to be taken into account. Small amounts of SDS will uncoil the storage proteins making them digestible but this does not hold with the extremely stable potato peroxidases. The stability of sap proteins facilitates the application of protein spectra in variety-diagnosis provided that the proteins are not affected by tanning reactions and alkaline solutions.

For gel electrophoresis we have applied a new Tris/borate-buffer pH 7.9 (along with the buffer pH 8.9) which contains a large amount of boric acid. This buffer proved to tolerate all of the ions found in the tuber tissue, particularly their changing amount, and sharp separation of protein in the

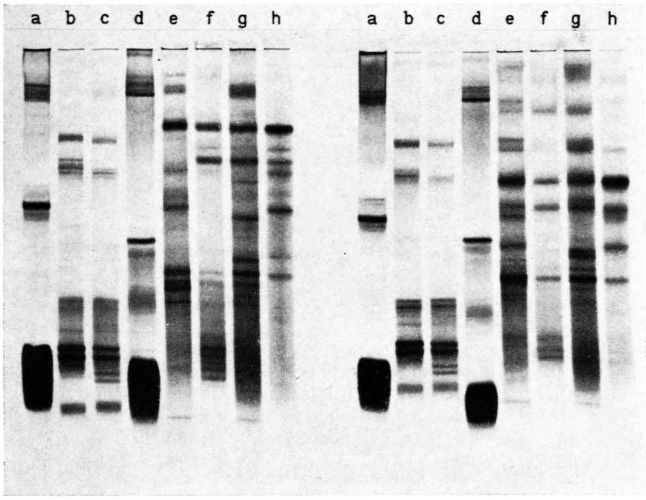

Fig. 1. Comparison of buffers pH 7.9 (left) and pH 8.9 (right). Electrophoresis in 5% Cyanogum at 500 V, 60–30 mA for 2 hours in both systems. In all figures anode at the bottom and gel stained with Supranolcyanin if not otherwise mentioned. For details: See Methods. Serum of (a) human, (b, c) trouts, (d) rabbit; concentrated preparations of potato tuber proteins, varieties (e) Lerche, (f) Lori, (g) Rosa, (h) Voran.

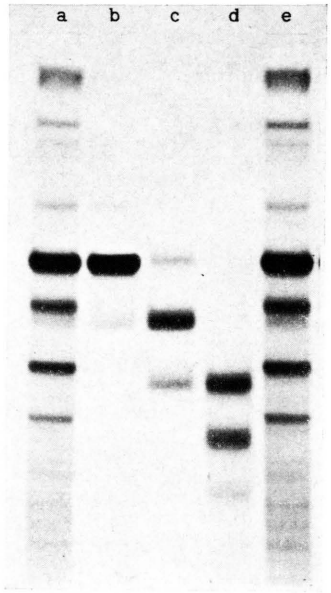

Fig. 2. Electrophoresis of one isolated fraction of Voran proteins before and after treatment at pH 10: (a, e) concentrated, unfractionated sample, untreated, (b) isolated fraction, untreated, (c, d) isolated fraction as in column (b) after treatment at pH 10 and 50 °C, for 2 and 20 hours, respectively. Electrophoresis at pH 8.9 as in Fig. 1.

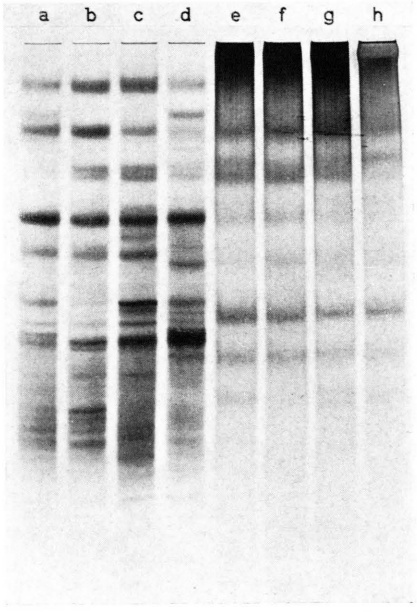

Fig. 3. Electrophoresis of concentrated samples from different varieties of potatoes (a–d) before and (e–h) after treatment at pH 10 and 50 °C for 16 hours. Varieties (a, e) Voran, (b, f) Maritta, (c, g)) Rosa, (d, h) Lerche. Electrophoresis at pH 8.9 as in Fig. 1.

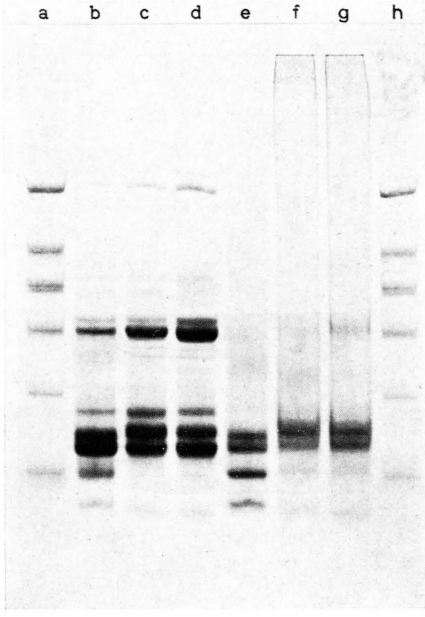

Fig. 4. Molecular weight determination (b–d) before and (e–g) after treatment at pH 10 and 50 °C for 16 hours. Electrophoresis of SDS/ME-treated proteins from (b, e) Voran, (c, f) Maritta and (d, g) Rosa in 5% gel, SDS/buffer pH 7.1, at 200 V, 90 mA for 3 hours; (a, h) marker proteins from top: Muscle phosphorylase b (92500), bovine serum albumin (67000), α-amylase from porcine pancreas (48200), alcohol dehydrogenase from yeast (37000), chymotrypsinogen A (25700), cytochrom c (12400).

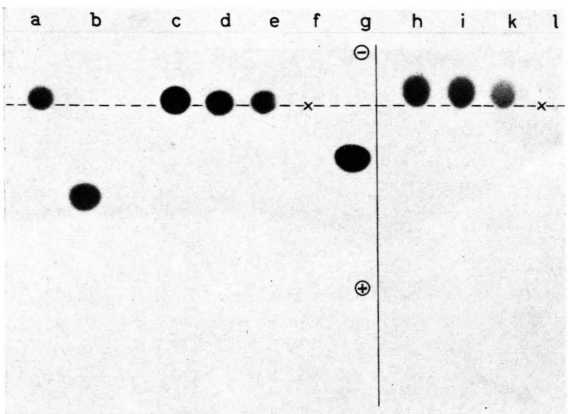

Fig. 5. Treatment of (a) DL-asparagine, (c—f) L-glutamine and (h—l) L-glutaminyl-L-glutamine at pH 10 and 50 °C: Incubation time (a, f, l) 24 hours, (e, k) 7 hours, (d, i) 4 hours; (b) aspartic acid (c) glutamine and (h) GLN-GLN as control, (g) glutamine treated as (f), followed by hydrolysis with 3 N HCL for 40 hours 50 °C, spot position identical with glutamic acid. Thin layer electrophoresis on cellulose MN 300 at pH 3.9 and 50 V/cm, 20 mA for 30 min according to [23].

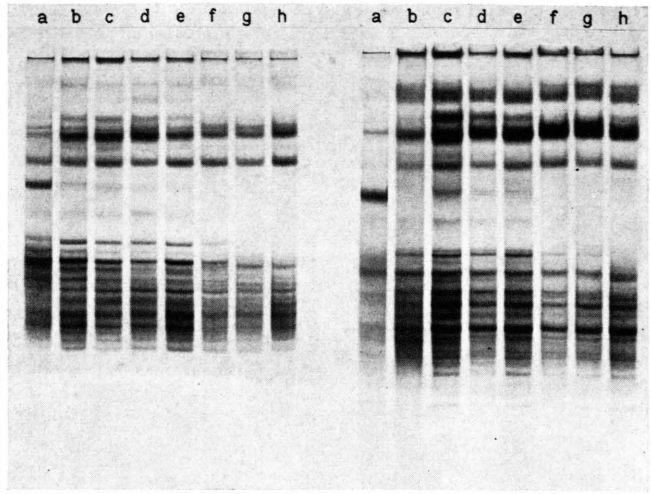

Fig. 6. Shift of the electrophoretic pattern of proteins taken from large tubers, variety Lori (left) and Maritta (right) during growth, storage and sprouting. (a—h) July—March, except December. Storage was between 6—12 °C; lower temperature would slow down the shift. Electrophoresis at pH 7.9 as in Fig. 1. Compare with Table I.

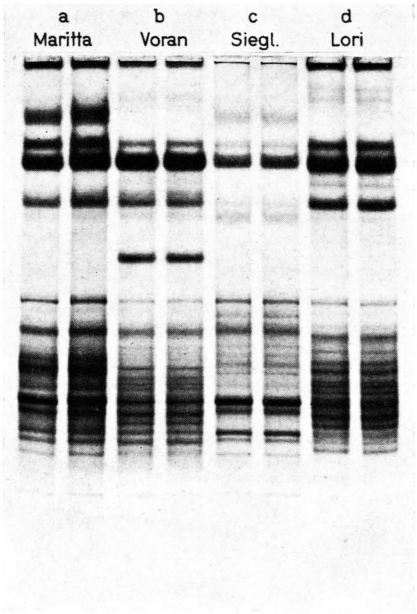

Fig. 7. Electrophoresis of proteins from potato tuber different in size. Crude sap from (a) Maritta, (b) Voran, (c) Sieglinde, (d) Lori. Electrophoresis at pH 7.9 as in Fig. 1.

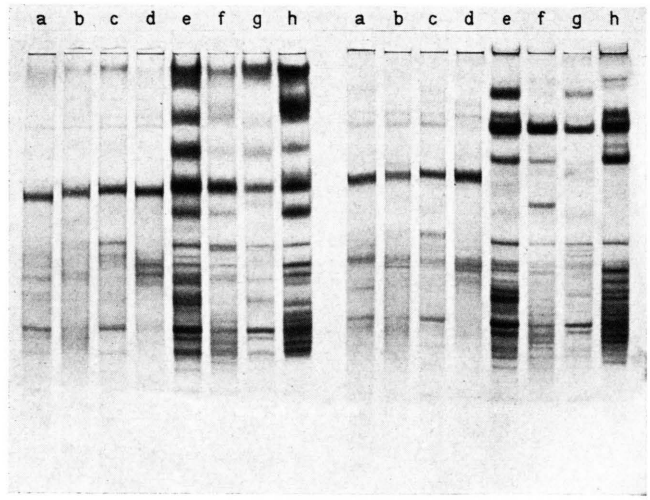

Fig. 8. Comparison of proteins from (a—d) immature and (e—h) mature potato tubers, harvested in July and October, respectively. Crude sap from (a, e) Maritta, (b, f) Voran, (c, g) Sieglinde, (d, h) Lori. Electrophoresis as in Fig. 1.

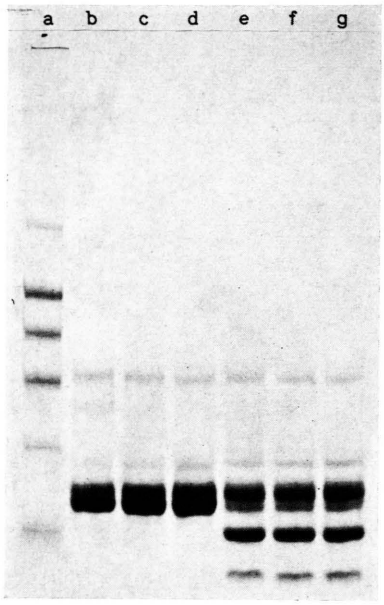

Fig. 9. Comparison of potato protein subunits (MW), variety Voran, obtained by SDS-treatment without or with reduction of S—S-bridges. (b—d) samples treated with 1% SDS at pH 7 and 37 °C for 20, 40 and 80 min, respectively; (e—g) samples treated with 1% SDS/1% ME under same conditions; (a) marker proteins as in Fig. 4.

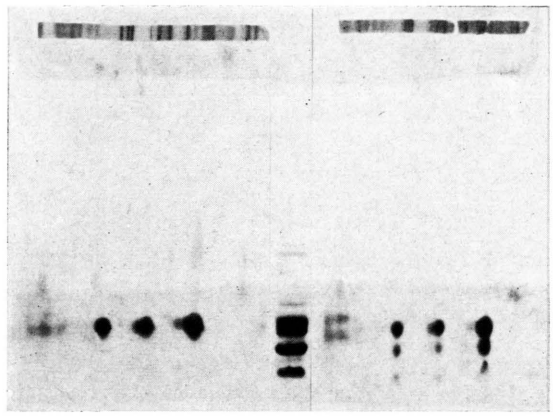

Fig. 10. Subunits (MW) of potato proteins, variety Voran, in relation to charge distribution of the proteins from the native sample, demonstrated by twodimensional separation in PAA-gel. 1. Dimension: Electrofocusing in tubes with Ampholine pH 5—7; 2. Dimension: SDS-electrophoresis as in Fig. 4; rods pretreated with SDS (left) or with SDS/ME (right) and positioned in the gel plate with the more acidic proteins towards the left. For more detailed description see text.

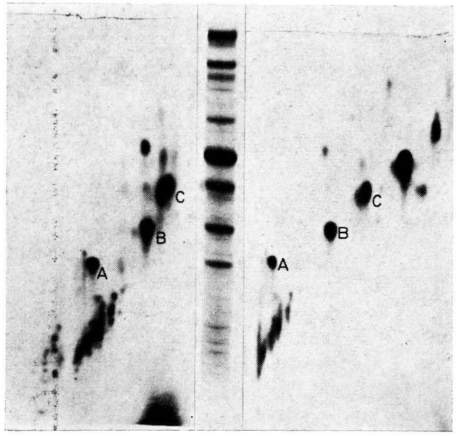

Fig. 11. Mapping of potato proteins, variety Voran. Identical spots marked. 1. Dimension: Electrofocusing in tubes with Ampholine pH 4—6 (left), pH 5—7 (right); 2. Dimension: Rod position as in Fig. 10, electrophoresis at pH 8.9 as in Fig. 1.

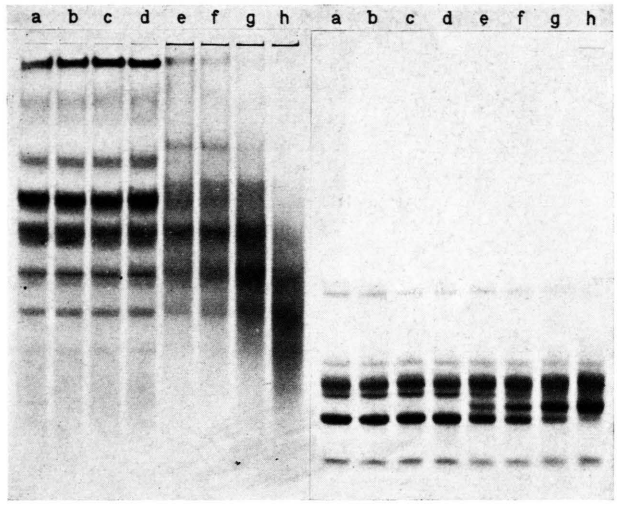

Fig. 12. Treatment of a concentrated sample of Voran with jodoacetamide followed by normal (left) and SDS/electrophoresis (right): (a—d) Controls, incubated at pH 8 and 25 °C without IAA for 1, 2, 4, 22 hours, respectively; (e—f) samples treated with IAA under same conditions, pH kept constant. (10 μl sample, corresponding to 200 μg protein, 5 mg IAA, and 25 μl Tris (1 M)/borate buffer pH 8; after incubation samples were dialyzed against the same buffer diluted 1:20 with distilled water.) Electrophoresis as in Fig. 1 and in Fig. 4.

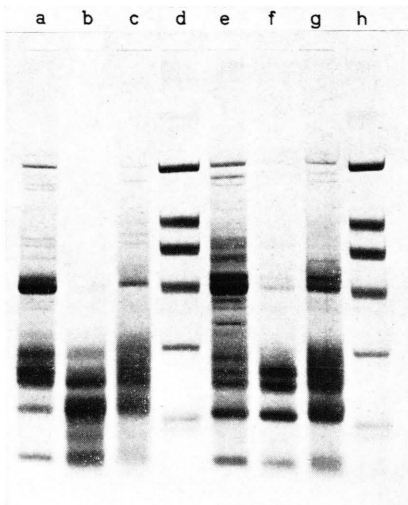

Fig. 13. Molecular weight determination of proteins from immature, mature and from cut slices of mature tubers. Varieties (a—c) Merkur, (e—g) Lerche; (a, e) immature, (b, f) mature tubers, (c—g) slices from mature tubers. Slices of 1 mm thickness were stored in Petri dishes for 16 hours at 20 °C, then frozen at —20 °C under addition of distilled water to restore the original weight of the sample. For sap preparation see text. (d, h) Marker proteins as in Fig. 4. Electrophoresis of the SDS/ME-treated proteins as in Fig. 4.

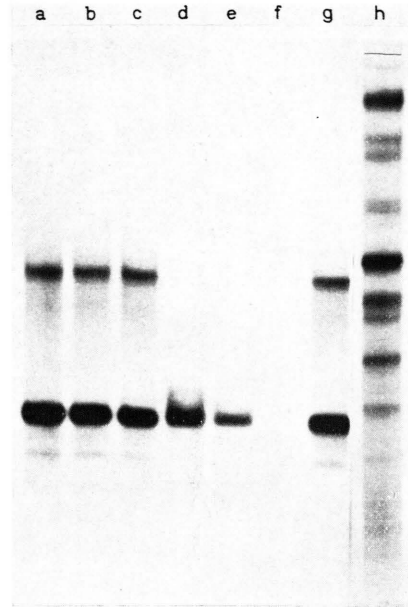

Fig. 14. Gradual unfolding of potato peroxidases (Voran) by SDS with increasing incubation time and temperature demonstrated by gradual loss of activity, staining with H_2O_2/o-dianisidine: (a, g) Control untreated; samples treated at pH 8 and 37 °C (b, c) without and (d, e) with 1% SDS for 3 and 16 hours respectively, (f) with SDS at 50 °C for 20 hours; (h) protein pattern of the untreated sample stained with Supranolcyanin. Electrophoresis without SDS at pH 8.9 as in Fig. 1.

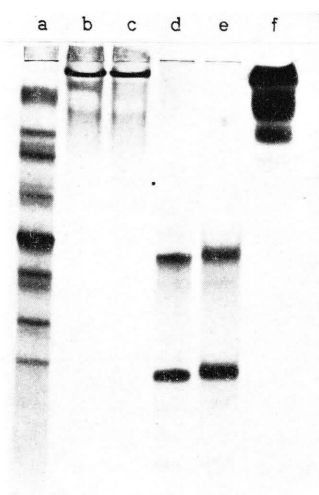

Fig. 15. Proteolytic action on proteins and peroxidases in sap of Voran and on horse-radish peroxidase: Bands stained for (a—c) proteins or (d—f) peroxidases; (a, d) Voran untreated, (b, e) 200 μg protein in 10 μl sap treated at pH 8 and 37 °C for 17 hours with 1 mg Nagarse (subtilopeptidase A, 3.4.4.16, Serva), (c) control with 1 mg Nagarse; (f) horse-radish peroxidase, treated with Nagarse as in (b, e). Electrophoresis at pH 8.9 as in Fig. 1.

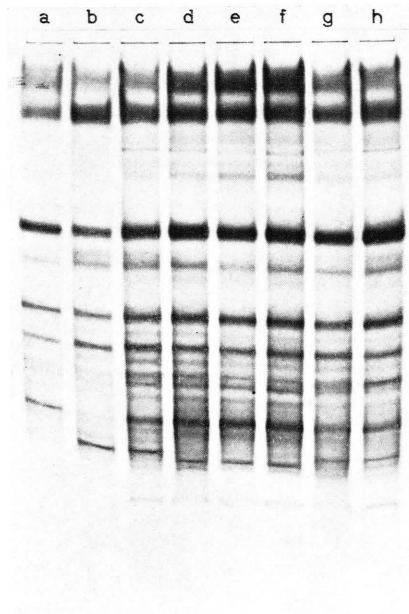

Fig. 16. Protein distribution in relation to sample sites. Crude sap from Feldeslohn: (a) total tuber; (b) skin; sections from (c) cortex zone, (d) vascular bundles, (e) pith tissure, (f) parenchymatic center, (g) stem end, (h) bud end. Electrophoresis at pH 8.9 as in Fig. 1.

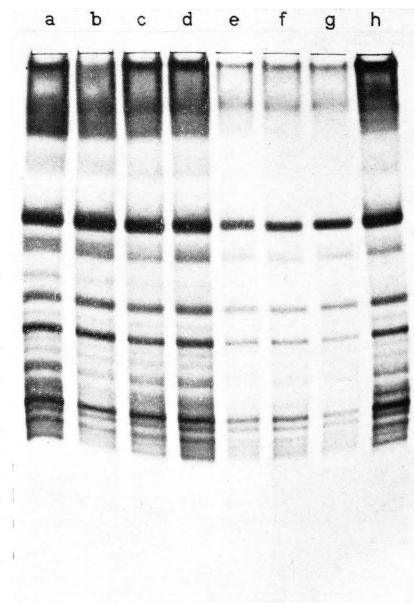

Fig. 17. Influence of herbicides on the protein distribution in tubers, variety Feldeslohn, investigated in (a—d, h) field and (e—g) pot trial: (a, e) Control, untreated, (b) Hedonal, (c) MH 30, (d, f) single and (g) double quantity of NCL, (h) Reglone. For more detailed description see text. Electrophoresis at pH 8.9 as in Fig. 1.

anodic range was achieved. There were no streaky or banded lines in electrophoresis and unfractionated sap could be applied with a higher load. Since most buffers increase their pH value with lower temperatures [30], the risk of deamidation is deminished. Finally the buffer pH 7.9 separated proteins well in the β- and γ-globulin region of trout serum * and could be used in almost all problems under investigation. As an example proteins of potatoes and 4 sera have been compared (Fig. 1). However, the load of sugars in the sample should not exceed 15%, otherwise borate complexes are formed [31] which disturb the even run in slab electrophoresis. If ready made gels are used, good storage quality is needed. Gels soaked with the buffer pH 7.9 for longer periods yielded almost the same protein pattern compared with freshly prepared gels in contrast to observations using the buffer pH 8.9. Discontinuous buffer systems were inconvenient and gave no better results. Among the dyes bromphenol blue up to 90 μg per 100 μl sample did not interfere with the proteins investigated in contrast to [32]. Some new versions of two-dimensional techniques with PAA as carrier have been introduced to differentiate between shifts of charge and size.

Protein spectra

We have tested roughly 200 potato varieties of the same physiological state and could not observe identical electrophoretic patterns among them. In one case where tubers of the variety Mensa were derived from 2 different meristemic cultures and displayed a divergent growth performance [33], the patterns were not to distinguish as well as the protein map [9] in the range pH 5 – 7, however, the map pH 4 – 6 showed different positions of very few spots and was extremely well reproducible.

Special attention was focused on the alteration of and relationship among all proteins in potato tubers caused by external or genetic factors. "All proteins" were defined as the stainable extracted proteins of the tuber which were surely a small fraction of all the individual proteins/enzymes present. Since the cork layer of the tuber had some proteins not common to the rest of the tuber and was rich in phenolic substances the skin was always discarded. Zacharius *et al.* [8] did not mention the removal of the peel and this and their use of electrophoresis in tubes instead of slabs may be partially responsible for their sometimes erratic results. In our experience the slab method is mandatory for experiments of this kind. Comparison of Fig. 7 and Fig. 8 demonstrates what can be expected under optimal conditions with respect to pattern identity in different runs.

The patterns do shift as long as the tubers are immature (Fig. 6). This is due to a change of the molecular weigths of the proteins and – to a greater extent – due to changes in charge. Electrophoretic spectra were constant for a given tuber from about August/September till spring if kept cool. Sprouting will again alter the pattern (Fig. 6), an observation in agreement with the findings of [8] and [4] and not quite in agreement with [2, 34]. One protein band disappears in all varieties when sprouting begins. As we have shown there are certain differences between early and late varieties. In spite of this the period of sample taking can be expanded from 3 months [4] up to 8 months, cool storage provided. The most interesting period for breeders and dealers in respect to variety identification is September through December. When checking immature tubers, *e. g.* harvested in spring in the mediterranean region, one has always to compare an authentic sample of the same state of maturity.

The pattern in July reflect a special case: the pictures from different varieties in the immature state resemble each other in general (Fig. 8). Since the main zone of the immature tuber has an almost identical mobility between pH 7.9 and 8.9 (no other pH-values tested) one must assume that an important part of the molecule supersedes typical characteristics of a protein. It is possible that here a protein is attached to a quite acidic carrier which may belong to acidic polysaccharides and that this part of the macromolecule is split off as maturity proceeds. This moiety was detected after staining by the Schiff's reagent as a strong band at the expected position.

Size distribution

The distribution and change of the MW of tuber proteins was investigated by gel electrophoresis in buffers containing SDS. SDS-loaded proteins are overcharged, thus only size determines the migration rates [11]. Most proteins in tubers belong to the

* In cooperation with Dr. Langholz and Dr. Keese, Göttingen.

MW-range 20 000, the protein MW 17 000 is split into smaller units by reduction of S – S-bridges, except when we were dealing with young tissue which contained little or no reducible bonds. In very young tubers of some varieties more dimers of the main proteins were found (see chapter "Properties and protein distribution"). In old tissue from sprouting (May, June) tubers the smaller subunits were predominant. We can regenerate the former pattern by induction of the embryonic state [35], by investigating slices of old tissue one day after slicing (Fig. 13). The change of zones after treatment with iodoacetamide was a further test that SH-groups were involved; the disappearance of the group of dimers means that these are probably linked by disulfide bonds.

It is obvious that the SDS-treated proteins represent a distribution no longer characteristic of the variety (Fig. 4). This means that the genetic background of storage proteins is mainly due to charge differences. Also an incubation at pH 10 which brings about deamidation will generate patterns less characteristic of the genetic origin (Fig. 3) affecting mainly the main proteins. This may signify that variations in the extent of amidation were responsible for the charge difference, whereby it is striking, that the newly formed zones in electrophoresis correspond often to zones genetically determined.

More convincing evidence was obtained by the determination of MW after charge separation by focusing (Fig. 10). The horizontal lines represent regions of the same MW and it is evident that in spite of different charges (pH 5 at left increasing to pH 7 to the right) the bulk of the proteins belongs to distinct MW-groups [36]. Two groups of MW 13 800 and 10 200 appear if a reduction by ME was carried through. The groups of proteins below 25 000 daltons show an almost identical charge distribution. When we determine the distribution of MW and the gel electrophoretic pattern after separation in a medium free of gel [18] with Ampholine of an even wider range (pH 5 – 10), the picture is similar. It gives an impression which zone of an electrophoretic pattern corresponds to which isoelectric point and MW respectively and how complex such a pattern is. The similarity of the size distribution and uniformity among the varieties gives a further hint that some of these proteins originated from one parent protein and that the diversity was developed later through charge specificity. The reliability of the MW-determination by the SDS-method is dependent on complete unfolding to the native primary structure, otherwise arbitrary MW will result. Peroxidases are an example where the usual incubation time [11, 26, 27] is not always sufficient. Thereby the activity stain indicates the coiled structure, the supranolcyanin stain serves as a nonspecific indicator. The storage proteins of potatoes behave normal, Fig. 14 shows that some peroxidases are very resistant to attack by SDS. Horse-radish peroxidase uncoils somewhat faster. When using the SDS-method, one should always test mobilities in different gel concentrations. The extrapolation to zero level of polyacrylamide should match a common intersect [37].

Alterations at pH 10

Midelfort and Mehler [38] have reported for the first time a deamidation of rabbit muscle aldolase *in vivo*. In the potato there could be a genetically controlled deamidation since some of the partially deamidated proteins from tubers of different varieties appear at the same position as proteins without treatment at pH 10. Treated fractions from an isolated zone show this phenomenon more clearly. Moreover the mobilities of extracts from proteins incubated at pH 10 are alike and independent of the original variety. Finally one should point out that protein patterns of very young tissue (Fig. 8) represent a nonspecific picture of the variety, they are rather similar which may mean, that the change in charge distribution (deamidation?) has a regulating function as discussed by Robinson *et al.* [39]. The share of generegulated synthesis de novo and a guided alteration later needs further elucidation. This problem is currently being investigated by fingerprints and serological tests. We have ruled out artefacts during preparation by repeated isolation procedures and by tracing the disappearance of GLN and GLN-GLN after incubation at pH 10 (Fig. 5). The kinetics for the hydrolysis of glutamine and asparagine are known [40] as well as the influence of positions and buffer ions [41, 42]. It is obvious that glutamine is faster deamidated than asparagine which is quite stable at pH 10.

The fast partial deamidation of proteins at pH 10 and in some cases already at pH 8 at room temperature should be ruled out always. We believe that this fact is often encountered in spite of more

optimistic views. It has been demonstrated [43] that focusing itself does not give rise to artificial bands. On the other hand isolation procedures are often tedious and bear the danger of hidden hydrolysis which may cause a misinterpretation of many patterns after electrofocusing. Since the amide group of glutamine is easily converted to the carboxyl group, this will give several differently charged proteins. Two-dimensional techniques are indispensable to identify an alteration like this.

Correlation between properties and protein distribution

There is no question that macrobiological properties are regulated on a molecular level. The sequence of amino acids in proteins composes a code which reflects the genotype and – in part – the net charge and size of proteins easy to determine. Assessment of genome relationships from protein spectra has been shown for wheat, *e. g.* [44, 45] and other cereals. For potatoes Desborough and Peloquin [46] have done interesting work on cultivars. Proteins of leaves are not very suitable in our and others experiences [47] if the plants were not grown and the leaves were not harvested under identical conditions. Also proteins active in photosynthesis are variable and major constituents. For the correlation of proteins in potato tubers to genotypes and prediction of properties it seems indispensable to know first the physiological changes. Nevertheless it has been demonstrated that in a potato which tends towards premature tuber formation (Knöllchensucht) the dimers of the 3 main proteins (in respect to MW) are more pronounced *e. g.* in the variety Merkur. Furthermore, early varieties change earlier from the general to the characteristic electrophoretic pattern.

More precise datas are not yet available to correlate the abundant polygenic properties with charge-, size- or gel-electrophoretic patterns of tuber proteins and enzymes. We are accumulating data in taking European collections of certified potato varieties for protein and isoenzyme analysis [29] and cooperate with geneticists [48] on mono- and diploid cultivars to learn about interrelationships of zones or spots after electrophoresis or mappings in respect to heredity and disease resistance.

We thank Ellen Krögerrecklenfort and Rosemarie Kaerger-Münder for their skilled assistance and Herrn K. Wieczorek for the precisely made equipment. We are indebted to the Bundesministerium für Landwirtschaft (Bonn) for financial help in the last part of the work, to Kali + Salz AG (Hannover) for supply of some chemicals, and to the Deutsche Forschungsgemeinschaft for a grant in 1969.

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