

Chromatography of Zein on Phosphocellulose and Sulfopropyl Sephadex

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ABSTRACT

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Whole zein (60% isopropanol-soluble) and α -zein (90% isopropanol-soluble) from corn endosperm were fractionated by ion-exchange chromatography on phosphocellulose and sulfopropyl Sephadex, respectively. Whole zein was dissolved in a lactate-ethylene glycol-isopropanol buffer (pH 3.8) containing mercaptoethanol, loaded on phosphocellulose columns, and eluted by a gradient of NaCl in the same buffer. The elution profile had 12 overlapping peaks. After α -zein was reduced and alkylated, it was loaded onto sulfopropyl Sephadex columns in a lactate-isopropanol buffer (pH 3.5) and eluted by a gradient of NaCl in

the same buffer. The elution profile included 10 broad peaks, most of which overlapped. The early-eluting peaks of whole zein and α -zein contained exclusively the smaller (mol wt 22,000) of the two major size components of zein, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas the late-eluting peaks contained both components. Isoelectric focusing analysis indicated that all peaks but one were heterogeneous and contained three to 12 charge species; however, one to three charge components predominated in each fraction.

Zein is the most abundant storage protein in corn endosperm, comprising 50–60% of the total protein. Polypeptides included in the zein fraction are unusual because they are insoluble in the aqueous buffer systems routinely used in protein isolation and purification, but they are readily soluble in aqueous alcohol solutions (eg, 60–70%, v/v, solutions of ethanol or isopropanol). The reason for this unusual behavior is that a preponderance of hydrophobic (leucine, alanine, proline) and uncharged polar (glycine) amino acid residues occurs in zein. Consequently, zein has virtually defied purification by conventional biochemical procedures. Attempts to fractionate zein by chromatographic procedures (Craine et al 1961, Fraij and Melcher 1978, Landry 1965) have in general not yielded satisfactory separations. No zein polypeptides have yet been purified to homogeneity. In a recent article (Esen 1980), I reported that zein polypeptides could be fractionated under nondissociating conditions by ion-exchange chromatography on phosphocellulose using buffers containing isopropanol (60%). This article is a detailed account of procedures and additional data.

MATERIALS AND METHODS

Preparation of Endosperm Meal

Kernels of a hybrid corn (WF 9X Bear 38) were soaked in water for 30 min, and endosperm tissue was isolated by removing the pericarp and germ from the kernel. Isolated endosperm preparations were rinsed with absolute methanol, air-dried, and ground in a Wiley mill to pass a 60-mesh screen. The resulting meal was further ground to a fine powder with a pestle and a glass mortar.

Zein Extraction

Zein was extracted from the endosperm meal with 60% isopropyl alcohol (IPA) by stirring in a 1,000-ml flask at room temperature (25°C) for 24 hr. A 50:1 solvent-meal (v/w) ratio was used. Zein was recovered in the supernatant after centrifugation at 20,000 \times g for 15 min. The extraction was repeated, but the second time, stirring for 24 hr was followed by heating in a water bath at 60°C for 30 min. Supernatants from both extractions were pooled and transferred to dialysis bags with a molecular weight cutoff of 8,000. Excess solvent was evaporated from the bags in a fume hood to reduce the extract volume to about half the original volume. After exhaustive dialysis against water, the turbid retentate and precipitate that formed in the bag were transferred to flasks and freeze-dried. Alternatively, pooled extracts were directly loaded

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onto the column after required quantities of the starting buffer components were added and the pH was adjusted.

Ion-Exchange Chromatography

Two cation exchangers, phosphocellulose (medium mesh, Sigma) and sulfopropyl (SP)-Sephadex (SP-Sephadex C-25, Pharmacia), were used. Phosphocellulose was washed before use with 0.5 M NaOH and 0.5 M HCl, respectively. The exchanger was then equilibrated with the starting buffer, and remaining fines were removed. The starting buffer was 0.01 M lactate (titrated to pH 3.8 with NaOH), which contained either 60% IPA or 45% ethylene glycol (EG) and 30% IPA. In addition, 2-mercaptoethanol (ME) at a final concentration of 0.1 M was included in the starting buffer in some experiments. SP-Sephadex was directly suspended in the starting buffer, equilibrated, and packed as described by the manufacturer. The starting buffer was 0.02 M Na-lactate (pH 3.5) containing 60% IPA. The reducing agent 2-ME was omitted because all separations on SP-Sephadex were with reduced, alkylated samples.

The columns were packed under gravity flow, and bed dimensions varied from 2.5 × 50 cm to 2.5 × 62 cm. The effluent pH was monitored before samples were loaded to ensure that the column was equilibrated with the starting buffer.

Some freeze-dried samples were dissolved in the starting buffer at a final concentration of 5 mg/ml and then were loaded onto the column. Others were first reduced, alkylated, dialyzed, and freeze-dried as described by Paulis and Wall (1977). These alkylated samples were extracted twice with 90% IPA, then sufficient water and buffer components were added to the pooled extract to bring the IPA concentration down to 60% before loading. Zein prepared in this manner is referred to as α -zein (Turner et al 1965) and includes the 22,000–24,000-dalton components. Columns were washed with three bed volumes of the starting buffer and developed with linear NaCl gradients. The range of gradients used routinely was either 0.0–0.1 M or 0.0–0.25 M, with the total gradient volume being four to eight times that of the bed volume. Ten-milliliter fractions were collected, and tube contents under each peak and subpeak were pooled. The pooled fractions were dialyzed against water and freeze-dried.

Electrophoretic Procedures

Chromatographic fractions were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for size heterogeneity and by isoelectric focusing (IEF) for charge heterogeneity. SDS-PAGE was performed according to the method of Laemmli (1970), except with 10–15% gradient gels (resolving gel). The resolving gel dimensions were 0.75 × 95–130 × 140 mm, whereas those of the stacking gel (5% polyacrylamide) were 0.75 × 15 × 140 mm. IEF was performed in 5% polyacrylamide gels (0.75 × 113 × 180 mm) containing urea at a final concentration of 6 M, as described by Gianazza et al (1976). Pharmalyte 5–8 (Pharmacia) was added to the gel mixture at a final concentration of 2% (w/v) to form the pH gradient. IEF was performed across the width on a Multiphor apparatus (LKB-Produkter). The anodic electrolyte solution was 0.04 M glutamic acid, and the cathodic solution was 0.1 M lysine (Caspers and Chrambach 1977). After IEF, the gel was placed in a glass dish containing H₂O and agitated for a few minutes to precipitate zein bands. Precipitated bands were viewed by holding the gel in water against a black background.

RESULTS

Chromatography of Zein on Phosphocellulose

All of the applied protein was bound to phosphocellulose under the starting conditions, indicating that polypeptides constituting zein had a net positive charge at pH 3.8. The breakthrough fraction contained some nonprotein material, such as pigments, absorbing at 280 nm. This fraction was devoid of protein when analyzed by dye-binding (Esen 1978) and showed no detectable protein bands when analyzed by SDS-PAGE. Development of the column with 0–0.1 M NaCl gradient yielded an elution profile with nine overlapping peaks (Fig. 1), some of which were poorly resolved.

When elution was continued with 0.1–0.3 M NaCl, three more poorly resolved, broad peaks were obtained. The protein that remained bound to the exchanger at 0.3 M NaCl concentration was eluted in one step with 1 M NaCl. Increasing ionic strength above $\mu = 1.0$ failed to permit desorption of any more protein. In fact, in a separate experiment, 0.5 M NaCl was sufficient to elute the fraction that remained bound at approximately $\mu = 0.3$. Development of the column with steeper gradients (0.0–0.5 M or 0.0–1.0 M NaCl) yielded elution profiles with fewer but sharper peaks. However, in these cases, resolution was not satisfactory because fractions representing different peaks had similar electrophoretic patterns.

Because zein polypeptides do not exhibit biological activity, SDS-PAGE and IEF were used to monitor the degree of purity in fraction pools representing different peaks. For example, nine fractions corresponding to peaks and subpeaks in Fig. 1, three fractions (10–12) representing peaks eluted with 0.1–0.3 M NaCl, and one fraction (13) eluted with 1 M NaCl were analyzed by SDS-PAGE (Fig. 3A). The early-eluting fractions 1–4 included one size component, the smaller (mol wt 22,000) of the two major zein components. In fact, these four fractions contained about 45% of the total protein recovered. Fractions 5–9 included primarily the two major size components of zein, and the late-eluting fractions (10–13) contained a low molecular weight component (15,000 daltons) in addition to the two major zeins. Fraction 13, eluted with 1 M NaCl, was made up primarily of one low molecular weight component (15,000 daltons). Furthermore, an additional size heterogeneity was repeatedly observed within each of the two major size components of zein. The larger component (mol wt 24,000) was made up of at least two size classes, as shown in profiles of fractions 8–12 (Fig. 3A and B). Similarly, the smaller major component (mol wt 22,000) included at least three size classes, as evident from the profiles of fractions 7–11 (Fig. 3A and B). Faint bands also existed in the 40,000–45,000-dalton region of SDS-PAGE gels. Moreover, SDS-PAGE data revealed that a given size component was common not only to adjacent fractions but also to those having widely separated elution orders (Fig. 3A).

Analysis by IEF revealed that the same fractions were all heterogeneous (Fig. 4A). The number of charge components varied from three to 12, depending on the fraction. In general, one to three charge components predominated in each fraction. All of the major charge components that occur in the IEF profile of unfractionated zein could be accounted for because the same components were present in one or more of the fractions. As observed in SDS-PAGE gels, polypeptides that were apparently the same occurred in IEF profiles of two to four fractions, which eluted in sequence. However, some charge components (Fig. 4A, arrows) were shared by many consecutive fractions (peaks), although they predominated in the profile of one or two fractions. Except for fraction 13, all fractions contained components with pIs (isoelectric

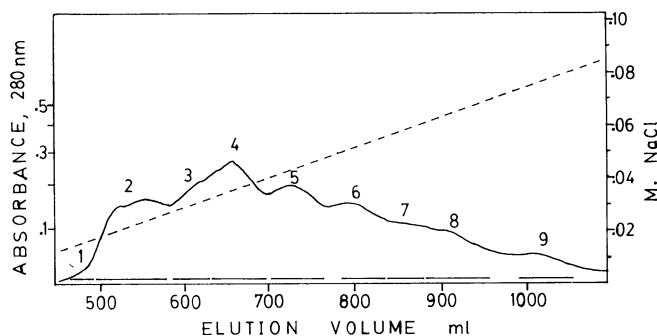


Fig. 1. Elution profile of reduced whole zein chromatographed on phosphocellulose. Only that part corresponding to elution with 0–0.1 M NaCl gradient is shown. Column bed, 2.5 × 62 cm; temperature, 24–25°C; buffer, 0.01 M Na-lactate in 45% (v/v) ethylene glycol, 30% (v/v) isopropanol + 0.1 M 2-mercaptoethanol, pH 3.8. Gradient volume, 1,150 ml. Broken line shows salt gradient. Bars above the abscissa indicate tubes pooled to constitute chromatographic fractions 1 through 9. Electrophoretic profiles of these fractions and others (10–13) are shown in Figs. 3A and 4A.

points) that appeared to be near or above seven. In fraction 13, all four major components obviously have acidic pIs because they focused towards the anodic end of the gel (Fig. 4A). However, pI measurements in the presence of urea are not reliable, and the experiment may not have been long enough for components to reach their apparent isoelectric point. In this study, IEF was used strictly as an analytical separation method to check the purity of fractions, not as a method for determining pI.

Chromatography of Zein on SP-Sephadex C-25

Alkylated samples containing α -zein components were run on SP-Sephadex. The development of the column with a 0–0.25M NaCl gradient yielded an elution profile with 10 broad peaks, most of which overlapped. Two early-eluting peaks (fractions) were devoid of protein, and thus they are not included in the elution profile shown in Fig. 2. No further protein was eluted with either 0.5M NaCl or 1M NaCl. When the column was eluted with a shallower gradient (0–0.1M NaCl), the overall elution pattern was similar to that in Fig. 2, but the peaks were much broader and less well-defined.

Analysis of the eight fractions corresponding to different peaks and subpeaks showed that the early-eluting fractions (1–4)

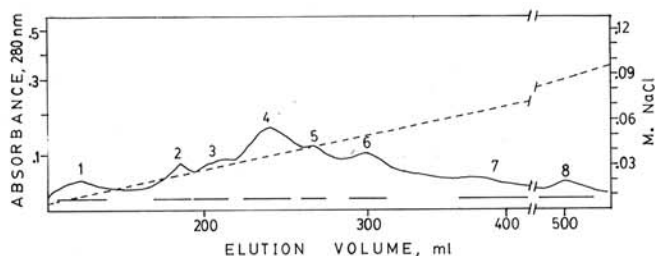


Fig. 2. Elution profile of reduced and alkylated α -zein chromatographed on SP-Sephadex C-25. Only the part of the profile with peaks that contain protein is shown. Column bed, 2.5 \times 54 cm; temperature, 24–25°C; buffer, 0.02M Na-lactate in 60% (v/v) isopropanol, pH 3.5; gradient, 0–0.25M NaCl; gradient volume, 1,180 ml. Broken line shows salt gradient. Bars above the abscissa indicate tubes pooled to constitute chromatographic fractions 1 through 8. Figures 3B and 4B show electrophoretic profiles of individual fractions.

contained the smaller (mol wt 22,000) of the two major size components of zein (Fig. 3C). In fact, the pattern of distribution of different size components within eight fractions was similar to that of the first nine phosphocellulose fractions. Thus, both cation exchangers appeared to yield comparable resolutions.

Likewise, IEF profiles of the SP-Sephadex fractions (Fig. 4B) generally resembled those of phosphocellulose fractions in heterogeneity and distribution of various charge components. The first two SP-Sephadex fractions displayed less charge heterogeneity than the corresponding phosphocellulose fractions. In fact, fraction one was essentially homogeneous, although the presence of a few other components in small quantities at higher sample loads could be demonstrated. One or a few charge components predominated the IEF profile of a given fraction, as with phosphocellulose fractions. The order in which different charge components eluted from the column usually was not consistent with their position in IEF gels.

Columns that were 50–60 cm long yielded better resolution than the columns that were 20–40 cm long. The shorter bed lengths yielded elution profiles with narrower, taller, but fewer peaks. However, the degree of separation of different size and charge components was not satisfactory, even though different components under different peaks were considerably enriched, as judged by IEF.

Both strong and weak anion exchangers (QAE-Sephadex and DEAE-Sephadex CL-6B, respectively) were used to further fractionate phosphocellulose and SP-Sephadex fractions. In both cases, most of the protein applied came through in the breakthrough fraction at pH 8.5 when 0.02M Tris-Cl containing 60% IPA was used as the starting buffer. When the bound protein was eluted with either linear or step gradients of NaCl, very little fractionation was achieved.

DISCUSSION

Results of this study clearly show that zein can be fractionated by chromatography on conventional strong cation exchangers when polar organic solvents (eg. alcohols) are included in buffers. Although neither of these exchangers was recommended for use with organic solvents, both appeared to withstand the conditions employed and could be used repeatedly upon regeneration. The

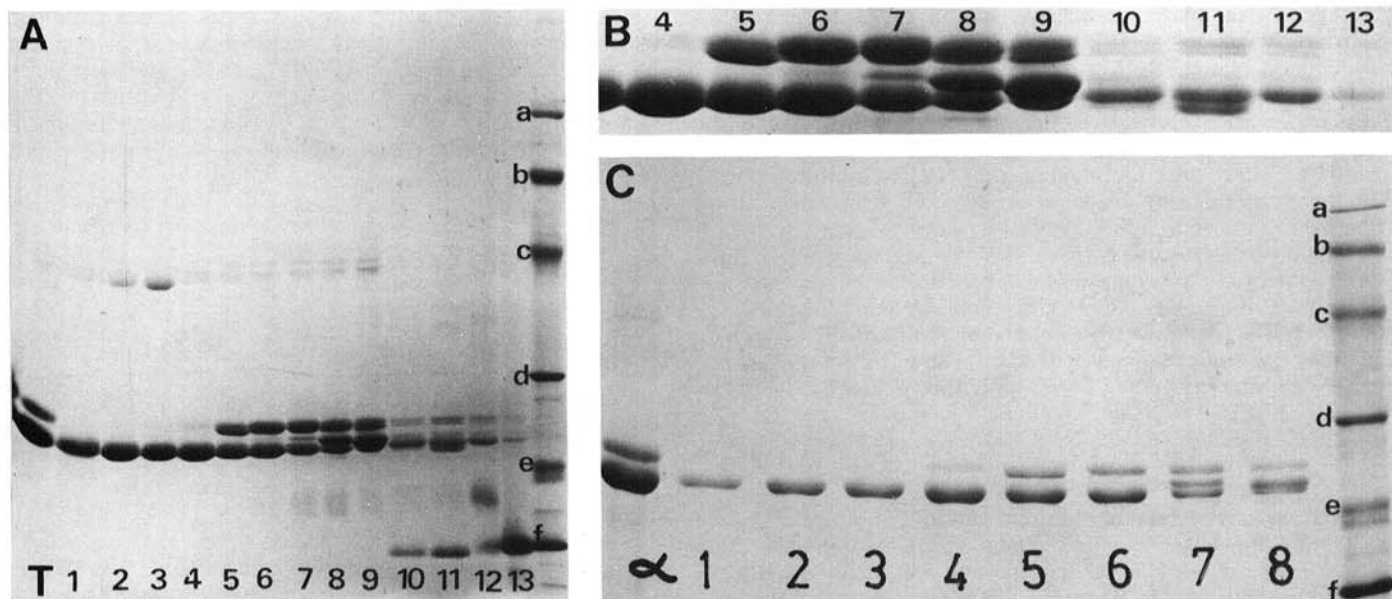


Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–15% gradient gel) patterns of fractions from chromatography of zein on two different cation exchangers, phosphocellulose, and SP-Sephadex. **Top**, cathode; **Bottom**, anode. Molecular weight calibration standards: a = phosphorylase b (94,000); b = bovine serum albumin (67,000); c = egg albumin (43,000); d = carbonic anhydrase (30,000); e = soybean trypsin inhibitor (20,100); f = hemoglobin monomers (16,100). A, molecular weight profile of total crude zein (track T) and phosphocellulose fractions 1–13. Fractions 1–9 were from elution with 0–0.1M NaCl; 10–12 eluted with 0.1–0.3M NaCl; and 13 eluted with 1M NaCl. B, magnification of the 20,000–25,000-dalton region of fractions 4–13 in A. C, molecular weight profile of total alkylated crude α -zein (track α) and SP-Sephadex fractions 1–8.

SDS-PAGE and IEF profiles of phosphocellulose and SP-Sephadex fractions indicated that the two exchangers were similar in resolution. The early-eluting SP-Sephadex fractions analyzed by IEF were more homogeneous than similar fractions from phosphocellulose. This may not necessarily indicate that the SP-Sephadex fractions had greater resolving power than phosphocellulose, because of differing chromatographic conditions, eg, pH, ionic strength of the starting buffer, and whether alkylated or nonalkylated samples were used. Phosphocellulose consistently gave much greater flow rates but exhibited more shrinkage than SP-Sephadex during gradient elution.

The most significant result was that both exchangers made it possible to obtain the smaller of the two major zein components in an essentially homogeneous state by size criterion, ie, a single band on SDS-PAGE gels (Fig. 3A, tracks 1-4; Fig. 3C, tracks 1-4). Yet, only the first of four fractions was homogeneous or nearly homogeneous by both size and charge criteria (Fig. 3A and C, track 1; Fig. 4A and B, track 1). Because each of these fractions was obtained by pooling individual tube contents under their respective peaks and subpeaks, the content of some individual tubes might have been homogeneous. Additionally, the total amount of protein recovered in these four fractions was 45% phosphocellulose to 60% SP-Sephadex. In other words, the 22,000-dalton component in gram quantities, which may be used for physicochemical characterization of this component, would be readily obtainable. In addition to the 22,000-dalton component, a low molecular weight component (15,000) was obtained in a nearly homogeneous state by the size criterion (Fig. 3A, track 13). This component may also be used for detailed physicochemical studies in its usual state or as the starting material for purification of its four charge components by other chromatographic or electrophoretic procedures. Because the larger of the two major components (the 24,000-dalton component) could not be separated from other size components, the purification of polypeptides in this size component might prove to be more difficult than others. However, preliminary experiments using closely spaced step gradients yielded fractions containing only the 24,000-dalton component (not shown).

Chromatographic separations were initially performed (Esen 1980) in the absence of a reducing agent. In this case, the peaks in the elution profile overlapped very little, but no significant fractionation of various size components was achieved. When 2-ME was included in the starting buffer, the trailing of the two major size components into the late-eluting fractions (peaks) disappeared, and the size and sharpness of these peaks decreased. Similarly, the early-eluting fractions were no longer contaminated with low molecular weight zeins, which began to appear consistently and exclusively under the late-eluting peaks whenever 2-ME was in the buffer. These results (Esen, unpublished data) indicated that a considerable proportion of zein polypeptides existed in disulfide-linked dimers, oligomers, and possibly polymers, as shown by Turner et al (1965). Even when alkylated samples were used or when the separations were performed in the presence of 2-ME, some trailing of the early-eluting 22,000-dalton component into the late eluting fractions still occurred. This fact and the lack of separation between the 24,000-dalton component and some 22,000 components were thought to be due to protein-protein association through hydrophobic interactions. Consequently, EG was included in the buffer to disrupt hydrophobic interactions, and some improvement in resolution was observed. However, whether this improvement was due to the disruption of hydrophobic interactions or to the decreased flow rate that occurs when EG is included is not known.

The occurrence of a given size component in fractions representing two or three consecutive peaks was expected because these adjacent peaks always overlapped one another. However, when the same size component appeared in fractions widely separated in their elution order, it was due either to the presence of different polypeptides with the same molecular weight or to anomalous trailing of the same polypeptide in too many fractions throughout the elution profile. A close examination of IEF profiles shows that some components with the same or similar pI (Fig. 4A,

arrows) occur in virtually every fraction, although they are primarily concentrated in two to three consecutive fractions; the order in which a given charge component elutes from the column is not always consistent with its apparent pI. In fact, the components contained in fraction 13 defied expectations. These components, apparently having lowest pIs, based on their position along the pH gradient, should have eluted in the earliest fractions. Yet, they required the highest salt concentration for desorption.

This anomalous elution pattern would have been attributed to the formation of zein dimers, oligomers, and polymers through intermolecular disulfide linkages and/or hydrophobic interactions if 2-ME and EG had not been included in the starting buffer. Therefore, other explanations must account for the observed anomalies in the elution pattern of some charge species, such as interactions other than ionic between proteins and the exchanger.

The existence of two subcomponents within the 24,000-dalton component has also been shown by Vitale et al (1980). However, three subcomponents within the 22,000-dalton component (Fig. 3B, track 11) were not unequivocally shown previously, although some indication of additional size heterogeneity was observed in *in vitro* translation products of zein mRNA (Viotti et al 1978, Park et al 1980). Apparently, the presence of a predominant size component within each size class masks other subcomponents in unfractionated samples. The separation of these predominant components from minor ones by chromatography and concentration of the minor components in certain fractions permitted their detection. Another reason for the detection of these additional size components was the use of polyacrylamide gel gradients having resolving power that was far superior to that of homogeneous gels. The occurrence of additional size heterogeneity suggests that at least five different mRNA size classes direct the synthesis of the 22,000-24,000-dalton zein polypeptides and that extensive charge heterogeneity of zein, reported previously by others (Gentinetta et al 1975), is accompanied by a greater size heterogeneity than was previously believed. Faint bands observed in the 40,000-45,000-dalton region of SDS-PAGE profiles were thought to be disulfide-linked zein dimers that escaped reduction by 2-ME.

The results reported here clearly show that zein polypeptides can be fractionated by ion-exchange chromatography. The strong cation exchangers followed by other chromatographic and electrophoretic procedures are very likely to yield homogeneous zein polypeptides. Purification of zein polypeptides to homogeneity and their thorough characterization, including primary structure, is of great interest to biochemists and evolutionary biologists alike. This is because comparative studies of amino sequences of prolamines within the genera *Zea* and

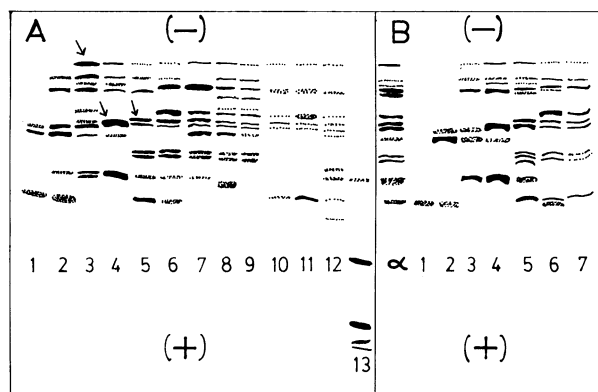


Fig. 4. Isoelectric focusing profiles (5% polyacrylamide gel, pH gradient formed with Pharmalyte 5-8) of fractions from chromatography of zein on two different cation exchangers, phosphocellulose, and SP-Sephadex. Profiles were directly traced to scale from wet gels in water. Broken lines and regions indicate faint bands. A, IEF patterns of phosphocellulose fractions 1-13. B, IEF patterns of SP-Sephadex fractions 1-7. The first track (α) shows α -zein pattern. Arrows indicate charge components occurring in many consecutive fractions.

Tripsacum may be the key to the elucidation of the origin of corn and of phylogenetic relationships between and within these genera. These chromatographic procedures are expected to apply to the fractionation of prolamines from other cereals.

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