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Preparation and rapid resolution of *Xenopus* phosvitins and phosvettes by high-performance liquid chromatography

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ABSTRACT

The presence of acidic phosivitins/phosvettes in *Xenopus laevis* yolk platelets and their purification by $(NH_4)_2SO_4$ precipitation of associated lipovitellin were documented by polyacrylamide gel electrophoresis followed by staining with Stains-all. Procedures were further developed to resolve the various entities present in the crude phosvitin/phosvette fraction by size-exclusion, anion-exchange, and hydrophobic interaction chromatography, using a Pharmacia FPLC system, and their resolution was documented by both electrophoresis and two-dimensional chromatography. Four major entities (phosvitins 1 and 2; phosvettes 1 and 2) were observed, but microheterogeneity was also apparent, particularly by hydrophobic interaction chromatography. The new separation procedures require min/h rather than h/days.

INTRODUCTION

"Phosvitins", first named in a classic paper by Mecham and Olcott [1], are highly acidic proteins found in vertebrate, but not invertebrate egg yolk [2,3], and are considered to bind and release calcium and other cations to the growing embyro [1,4–6]. About half of all phosvitin amino acids are phosphoserine, and the reported size of these proteins ranges from 30-40 kDa [5,6]. Smaller, phosvitin-like proteins, termed, "phosvettes" [7], have also been described from the ovaries of *Xenopus laevis* [7] and the eggs of the chicken [8,9].

Phosvitins and phosvettes are derived from vitellogenin, which is synthesized and secreted by the liver and transported via the maternal bloodstream to the growing oocyte, where it is sequestered and preteolytically processed into the various yolk proteins [10]. Multiple vitellogenins apparently exist for several vertebrate species [11–13], so it is presently uncertain whether phosvettes are derived from phosvitins that have sustained an additional cleavage during the proteolytic processing of vitellogenin [8] or from parental vitellogenins with smaller phosphorylated domains as recently described for chicken vitellogenin III [14]. The complete amino acid sequence of chicken vitellogenin II has been published [15] and sufficient N- and C-terminal information existed to allow the amino acid sequence assignment of the "major"

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phosvitin derived from this precursor [16,17]. In *X. laevis,* four vitellogenins are produced by the liver [18] and the amino acid sequence of one of these (vitellogenin A2) is known [19]. However, no comparable information has thus far been obtained for any of the derived *X. laevis* phosvitin/phosvette sequences. With a view towards this goal, we have reinvestigated the isolation procedures for *X. laevis* phosvitin/phosvettes.

Although the phosvitins/phosvettes are relatively easy to separate from other yolk proteins [20], their subsequent indication and resolution from one another is challenging because they absorb poorly or not at all at 280 nm [1,7,9,21–25], they do not react with anionic dyes such as Coomassie blue [7,8,25,26], and they have a very low isoelectric point and highly negative surface charge [27–30]. A previous study of *X. laevis* yolk platelets [7], relying primarily on analysis for ^{32}P-labeled proteins, demonstrated the presence of two phosvitins and two phosvettes. The phosvitins and phosvettes, and subsequently the two phosvettes, could be separated from one another by low pressure chromatographic methods [7]. We here report on the resolution and isolation of *X. laevis* phosvitin/phosvettes using chromatographic and desalting procedures that take several hours rather than several days. These procedures are adapted from methods originally developed for the chicken phosvitin/phosvettes [8,9].

EXPERIMENTAL

Animals and reagents

Adult female *X. laevis* were obtained from Nasco (Oshkosh, WI, U.S.A.) and fed a diet of frog brittle three times a week prior to use. Ultrapure $(NH_4)_2SO_4$ was obtained from Research Plus (Bayonne, NJ, U.S.A.). All other chemicals, including 1-ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naphtho-[1,2-*d*]thiazolium bromide (Stains-all), were purchased from Sigma (St. Louis, MO, U.S.A.).

Phosvitin/phosvette preparation

Females were anesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester (tricaine) for 30 min; the ovaries were excised, rinsed in 10% amphibian Ringer's solution, and cut into small pieces. The pieces were homogenized in two volumes of medium [0.25 *M* sucrose, 10 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.5), 1 m*M* EDTA, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1% polyvinylpyrrolidone]; the homogenate was then filtered through gauze and centrifuged at 500 *g* for 5 min. The pellets were repeatedly (3–4 times) resuspended in two volumes of homogenization medium and recentrifuged until light-green pellets of yolk platelets and clear supernatants were obtained. The platelets (which could be stored for several days) were then dissolved in 5 ml (per ovary) 1 *M* NaCl [containing 10 m*M* HEPES (pH 7.5) and 2 m*M* PMSF], the solution centrifuged in a microcentrifuge (13 600 *g*) for 15 min to remove a small amount of insoluble matter, and two volumes at 0°C saturated $(NH_4)_2SO_4$ were slowly added with stirring to precipitate the lipovitellins [20], which were removed by centrifugation. The supernatant, which contained the phosvitin/phosvettes, was passed (2.5 ml at a time) over a column (50 ml bed volume) of Bio-Gel P-6DG (Bio-Rad) previously equilibrated with water. The crude phosvitin/phosvettes, which elute in the void volume [9], were pooled and

lyophilized. The average yield from one ovary was 50–60 mg (elemental analyses: N = 14.1%; P = 7.7%). All operations were performed at 0–4°C. PMSF was initially prepared as a 200 m*M* solution in ethanol, stored at 4°C, and diluted 100-fold immediately prior to use, yielding slightly cloudy solutions; inclusion of PMSF in the ovary homogenization and platelet dissolution steps is necessary and sufficient to prevent inadvertant proteolysis of the phosphoproteins.

Chromatography

All chromatographic manipulations were performed on a Pharmacia FPLC system equipped with an automatic injector, a dual chart recorder, a 10-ml Superloop, dual monitors (214 and 280 nm) containing high-resolution flow cells, and a fraction collector. Three Pharmacia Superose columns ("12 + 12 + 6"; 300 × 10 mm I.D. each) were used in series for size-exclusion chromatography (SEC), an analytical Pharmacia Mono Q column (74 × 5 mm I.D.) for anion-exchange chromatography, and an analytical Bio-Rad Bio-Gel TSK phenyl-5-PW column (75 × 7.5 mm I.D.) for hydrophobic-interaction chromatography (HIC). All solutions pumped through the columns were initially filtered through 0.2-μm pore filters and degassed by sonication. Protein samples were injected onto the columns through 0.45-μm pore nylon syringe filters (Micron Separations). Sequential fractions eluted from the Superose columns were either dried in a vacuum centrifuge and prepared for gel electrophoresis, or further analyzed via the Superloop by anion-exchange and HIC. Sequential fractions eluted from the Mono Q or TSK phenyl-5-PW columns were individually desalted via the automatic injector on a column (280 × 16 mm I.D.) of Bio-Rad Bio-Gel P-6DG equilibrated with water, evaporated to dryness in a vacuum centrifuge, and prepared for gel electrophoresis.

Gel electrophoresis

Protein samples and column effluents (after desalting) were dissolved in sample buffer, electrophoresed on linear gradient (6.9–20.4% polyacrylamide) slab gels, and stained with Stains-all and/or Coomassie blue as previously described [8,25,31]. Protein size estimates were made according to Lambin [32,33], using Bio-Rad sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) standards.

RESULTS AND DISCUSSION

Yolk platelet fractionation

$(NH_4)_2SO_4$-Precipitation of lipovitellin from yolk protein mixtures was originally suggested as a general procedure for obtaining phosvitin from a variety of vertebrate eggs [20]. More recently, we have found this procedure to be effective for both *X. laevis* yolk platelets [7] as well as chicken yolk granules [8]. In the former case, fractionation of [^{32}P]protein was monitored, and it was discovered that if appropriate care was taken, [^{32}P]phosphoproteins smaller than phosvitin (and termed "phosvettes") could also be recovered from the $(NH_4)_2SO_4$ supernatant [7]. Phosvettes from chicken granules have also been detected, along with the usual phosvitins, by staining electrophoretic gels with Stains-all [8].

We here document by this staining procedure that $(NH_4)_2SO_4$-precipitation of lipovitellins from dissolved *X. laevis* yolk platelets can be used to recover in un-

contaminated form essentially all acidic polypeptides present in the original yolk platelets (Fig. 1). Yolk platelets and protein remaining in the $(NH_4)_2SO_4$ supernatant derived from *X. laevis* yolk platelets were analyzed by gradient gel electrophoresis followed by staining with Stains-all and/or Coomassie blue (Fig. 1). Stains-all failed to stain the size-calibration proteins used (Fig. 1a, lane 1), but indicated a major doublet at 30–31 kDa, a minor band at 24 kDa, and a band at 13 kDa in both the yolk platelets and $(NH_4)_2SO_4$-supernatant fraction (Fig. 1a, lanes 2 and 3, respectively). When this same gel was subsequently stained with Coomassie blue, the molecular size markers were revealed (Fig. 1b, lane 1) as well as some additional proteins present in the yolk platelets (Fig. 1b, lane 2), but no additional bands were found for the $(NH_4)_2SO_4$ supernatant (Fig. 1b, lane 3). The additional bands present in yolk platelets were revealed more clearly when Coomassie blue alone was used as a stain and consisted of unresolved large lipovitellin subunits at 110 kDa and small lipovitellin subunits at 29–31 kDa (Fig. 1c, lane 2) [7]. Again, these were absent from the $(NH_4)_2SO_4$-supernatant fraction (Fig. 1c, lane 3). We conclude that the acidic proteins (phosvitin/phosvettes) present in yolk platelets are retained in the $(NH_4)_2SO_4$-supernatant fraction, are uncontaminated by Coomassie blue-staining proteins (lipovitellins), and thus serve as adequate starting material for subsequent chromatography.

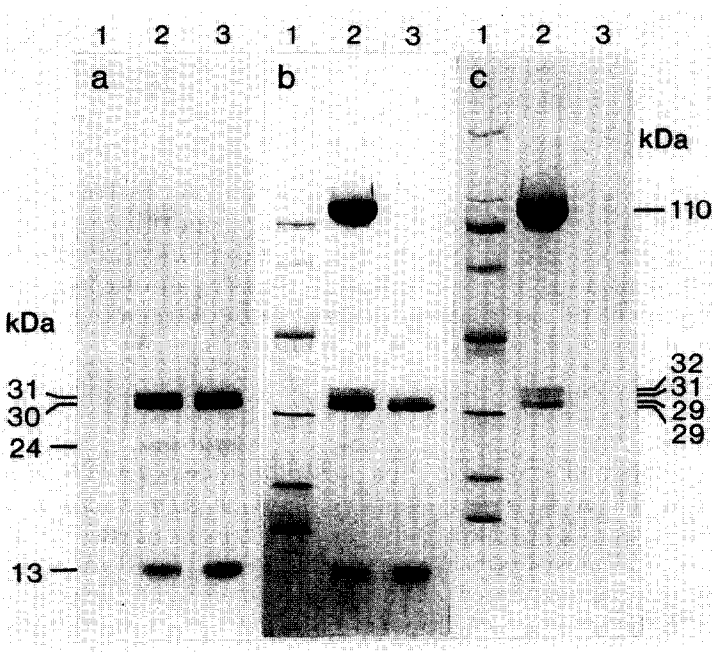

Fig. 1. Gradient polyacrylamide gel electrophoresis of (lane 1) molecular weight standards, (lane 2) approximately 100 μg *X. laevis* yolk platelets, and (lane 3) approximately 20 μg crude *X. laevis* phosvitin/phosvette fraction. Gels were stained with (a) Stains-all, (b) Stains-all plus Coomassie blue, or (c) Coomassie blue. The patterns indicated in (a) and (b) are from the same gel, while (c) represents a second gel. Some fading of bands indicated by Stains-all occurs during processing with Coomassie blue.

Exploratory chromatographic attempts

A variety of chromatographic procedures were examined to resolve the acidic components present in yolk platelets. Nothing adsorbed to a cation exchange column (Pharmacia Mono S), while the various components present adhered to a chromatofocusing column (Pharmacia Mono P) very tightly and could only be removed with an acid wash. Similarly, a hydroxyapatite column (Bio-Rad Bio-Gel HPHT) column failed because the phosphoproteins present could only be partially eluted with very high concentrations (> 1.5 *M*) of phosphate buffer. Reversed-phase chromatography (Pharmacia ProRPC) was also unsuccessful: 100% acetonitrile (with 0.1% trifluoroacetic acid) failed to elute adhered components, while a gradient of methanol (with 0.01 *M* triethylammonium acetate, pH 6.3) eluted the various components together. Successful resolution of the phosvitin/phosvette components was only achieved with the three chromatographic procedures that follow.

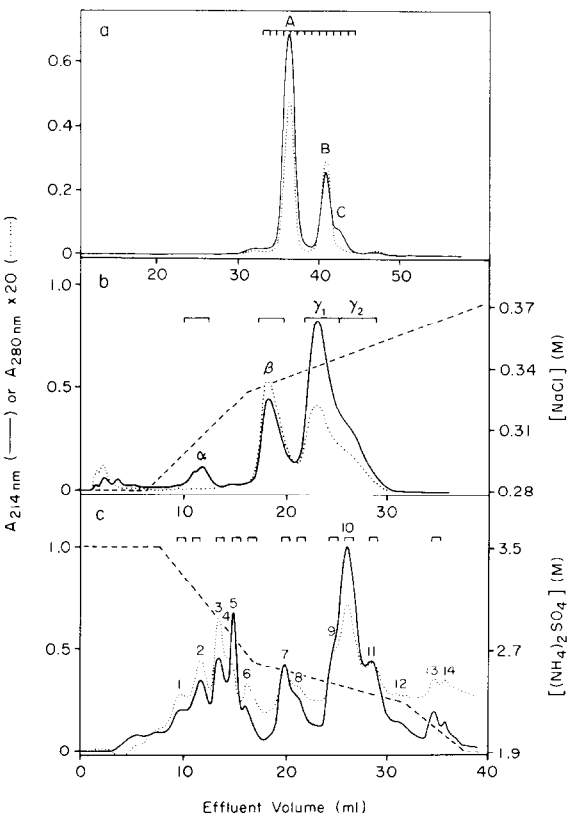

Fig. 2. Resolution of the crude *X. laevis* phosvitin/phosvette fraction by (a) size-exclusion, (b) anion-exchange, and (c) hydrophobic interaction chromatography. Approximately (a) 0.2 mg, (b) 0.5 mg, and (c) 1.1 mg of the $(NH_4)_2SO_4$-soluble fraction from yolk platelets were applied to each column and eluted with (a) an isocratic solution of 0.05 *M* $(NH_4)HCO_3$, (b) an increasing gradient of NaCl in 0.05 *M* Tris–HCl, pH 7.5, and (c) a decreasing gradient of $(NH_4)_2SO_4$ in 0.1 *M* sodium phosphate, pH 6.8. Flow-rates were 0.5, 1.0, and 0.5 ml/min, respectively. Fractions collected for subsequent electrophoretic analysis are indicated at the top of each chromatograph.

SEC of the phosvitin/phosvette fraction

SEC of the crude phosvitin/phosvette fraction in 0.05 *M* NH_4HCO_3 indicated the presence of at least three components, designated A, B and C (Fig. 2a) in order of their elution position. The putative size of each component, calculated with the aid of Bio-Rad native calibration standards, is 222, 86, and 62 kDa, respectively. The relative amounts of components A–C (based on total 214 nm absorbance eluted from the columns) are 68%, 21%, and 7%, and the 280/214 nm absorbance ratios for each component are 0.03, 0.05 and 0, respectively. Gradient gel electrophoretic analysis of sequential eluted fractions demonstrated that component A primarily comprises the doublet at 30–31 kDa, although some 24- and 13-kDa phosphoproteins are also present in this peak, while components B and C represent the 13- and 24-kDa phosphoproteins, respectively (Fig. 3).

When we used salt concentrations greater than 0.05 *M*, all components eluted at later times; this effect was more pronounced for components A and B than for component C, with the result that components B + C merged and migrated as a single peak (data not shown). The pronounced dependence of elution position on ionic strength, previously documented for the chicken phosvitin/phosvettes and thought to be due to their high surface charge [10], precludes an accurate size determination of phosvitin/phosvettes by SEC, and the nominal values we have calculated (62 222 kDa) are thus spuriously high. However, the less pronounced effect of ionic strength on the elution position of component C would suggest that it has a lower surface charge, and hence may be less phosphorylated, than components A and B. This notion is reinforced by finding that components A, B, and C comprise the 31 + 30, 13, and 24 kDa bands, respectively, as indicated by gradient PAGE (Fig. 3). Thus,

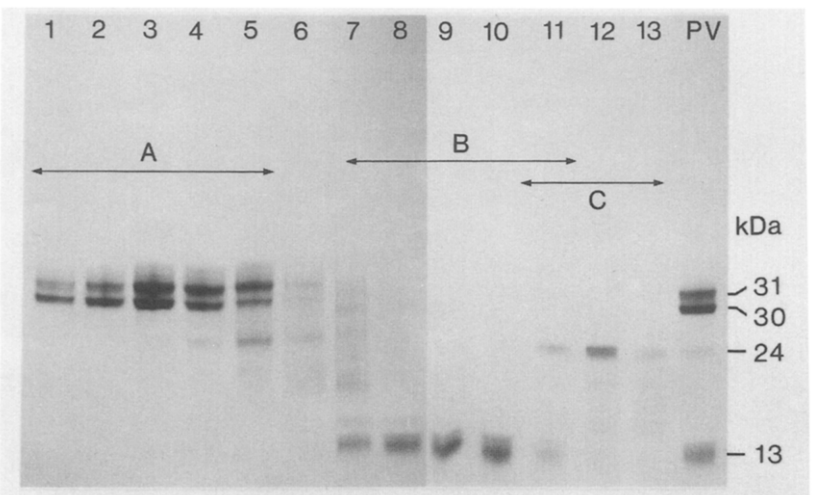

Fig. 3. Gradient gel electrophoresis of fractions obtained by SEC. Approximately 1.3 mg of a crude *X. laevis* phosvitin/phosvette fraction was chromatographed as in Fig. 2a and individual 0.7-ml eluent fractions, representing components A–C, were dried in a vacuum centrifuge and taken up with 400 μl sample buffer each. The volumes of sequential samples added to lanes 1–13 were 10, 5, 5, 5, 5, 17, 50, 25, 8, 5, 10, 30, and 35 μl, respectively; the last lane (PV) represents approximately 25 μg of the original crude phosvitin/phosvette.

components A and B elute from the Superose columns unusually early, relative to component C, indicating a greater charge repulsion between these early-eluting components and the column matrix.

Anion-exchange chromatography of the phosvitin/phosvette fraction

Anion-exchange chromatography again revealed the presence of at least three populations, labeled α, β, and γ in order of their elution by an increasing NaCl gradient (Fig. 2b). The α- and γ-populations both displayed the presence of subpopulations. The relative abundance and the 280/214 absorbance ratios for the three populations were 7, 24 and 64%, and 0, 0.05, and 0.03, respectively. Gel electrophoretic analysis of the three populations (together with the 280/214 ratios) indicated that the α-, β-, and γ-populations represented 24-, 13-, and 30 + 31-kDa material, respectively (Fig. 4). No major differences were apparent between the early (γ_1)- and late (γ_2)-eluting portions of the γ-population (Fig. 4). Anion-exchange chromatography thus elutes the phosvitin/phosvettes in reverse order from that obtained by SEC. The resolution of the α- and β-populations (Fig. 2b), however, is much greater than that achieved for components B and C by SEC (Fig. 2a).

HIC of the phosvitin/phosvette fraction

HIC with relatively high concentrations of $(NH_4)_2SO_4$ provided the most complex elution pattern. At least 14 peaks were discerned in the eluate after initializing a decreasing $(NH_4)_2SO_4$ gradient (Fig. 2c). Pattern complexity and a shifting 280-nm baseline precluded accurate relative abundance and absorbance ratio calculations. In

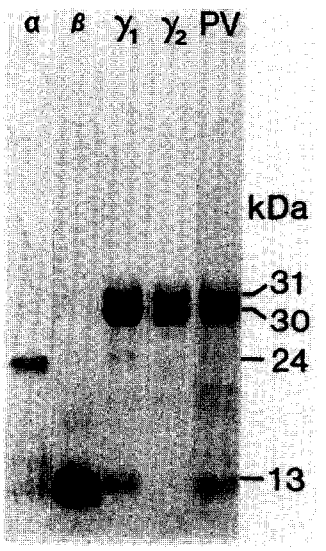

Fig. 4. Gradient gel electrophoresis of fractions obtained by anion-exchange chromatography. Approximately 1.0 mg of the crude *X. laevis* phosvitin/phosvette fraction was chromatographed as in Fig. 2b and 2-ml (α and β) or 3-ml (γ_1 and γ_2) fractions were diluted with equal volumes of double-strength sample buffer. The volumes added to each lane were 20 μl in each case; the PV lane contained 20 μg of the original crude phosvitin/phosvette.

general, however, relatively high 280/240 ratios were observed for the early-eluting peaks (Nos. 1–4, 6), while lower ratios were displayed by late-eluting peaks (Nos. 7–14). Peak 5 appeared to have little or no absorbance at 280 nm. Gel electrophoretic analysis of the various peaks indicated that the early-eluting peaks comprised 13-kDa material, with the exception of peak 5 which was enriched with the 24-kDa phosphoprotein, while the later peaks comprised members of the 30 + 31-kDa doublet (Fig. 5). Some indication also exists that the later eluting peaks comprise pure populations of either the 31- or 30-kDa bands. However, an alternate electrophoretic procedure, which better resolves these two bands, will have to be developed before this can be convincingly demonstrated.

The reason for the complexity indicated by HIC is unknown. A similar complexity was observed for chicken yolk granule phosphoproteins [9] and, as found for the numerous chicken phosphoprotein peaks, each major peak of *X. laevis* material rechromatographed in its original position (data not shown), thus suggesting integral populations. We have previously suggested that multiple conformational states may be induced by the high concentration of $(NH_4)_2SO_4$ in which the samples are applied to the column [9,34], with the integral populations being dependent on threshhold levels of a post-translational process such as phosphorylation or glycosylation. Various subpopulations of the same polypeptide would nevertheless co-electrophorese as a single band in the presence of SDS and a low ionic strength buffer.

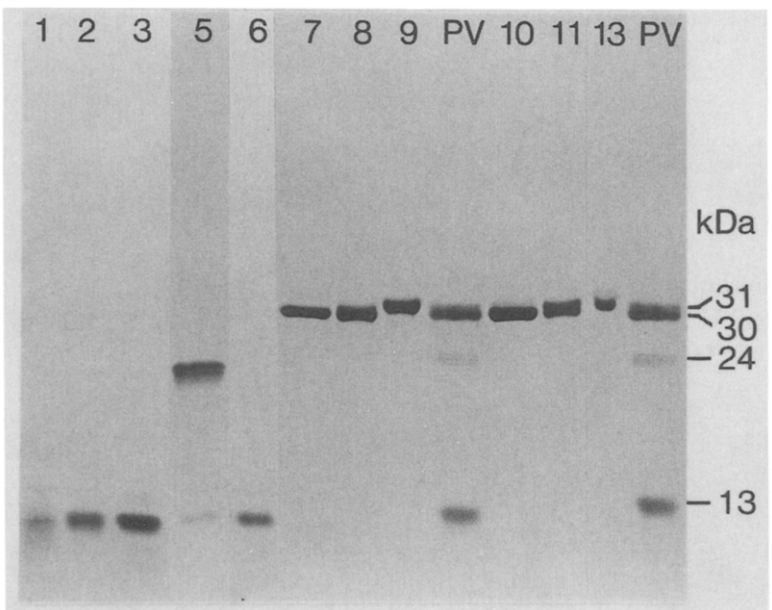

Fig. 5. Gradient gel electrophoresis of fractions obtained by HIC. Approximately 0.5 mg of the crude *X. laevis* phosvitin/phosvette fraction was chromatographed as in Fig. 2c and individual 0.7-ml fractions, representing all the peaks except Nos. 4, 12, and 14, were desalted on a Bio-Gel P-6DG column, dried in a vacuum centrifuge and taken up with 100 μl sample buffer each. The sample volumes for the indicated peaks were 60, 20, 15, 12, 20, 15, 20, 15, 7, 15, and 40 μl, respectively; the PV lanes contained 20 μg of the original crude phosvitin/phosvette.

Two-dimensional chromatographic analyses of the phosvitin/phosvette fraction

In order to help cross-reference the various components revealed by each column method, the phosvitin/phosvette fraction was initially subjected to SEC, and eluant fractions were subsequently analyzed by anion-exchange chromatography and HIC. Secondary anion-exchange chromatography (Fig. 6) indicated that component A gave rise to the τ-population, with the initial part of component A being enriched in late-eluting τ-material. Component B comprised the β-population, while component C consisted of the α-population. Secondary analysis by HIC proved to be more complex (Fig. 7). Component A gave rise in succession to peak 9 and a small amount of previously unidentified material, followed by peaks 7, 10, and 13 together, and then peaks 3, 6, 8, 11, 12, and 14 together; component B initially produced peak 1 followed by peaks 2, 3, and 6 together; finally, component C comprised only the peak-5 material.

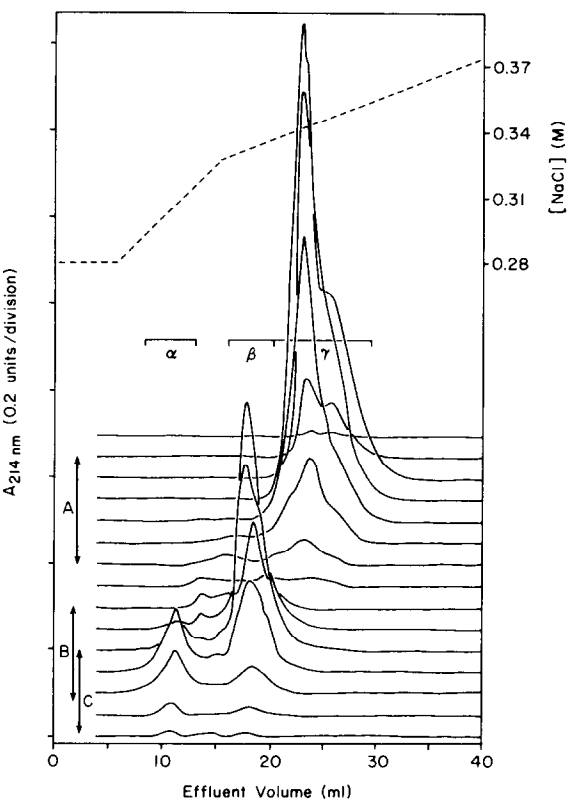

Fig. 6. Anion-exchange chromatography of fractions obtained by SEC. Approximately 2.0 mg crude *X. laevis* phosvitin/phosvette was chromatographed as in Fig. 2a, but using 0.05 *M* Tris–HCl, pH 7.5, containing 0.28 *M* NaCl as the eluting buffer with a flow-rate of 0.2 ml/min. Individual 0.7-ml eluent fractions, representing components A–C, were directly applied to a Mono Q column via the Superloop, and eluted with an increasing concentration of NaCl (indicated for the effluent) in 0.05 *M* Tris–HCl, pH 7.5, at a flow-rate of 1.0 ml/min.

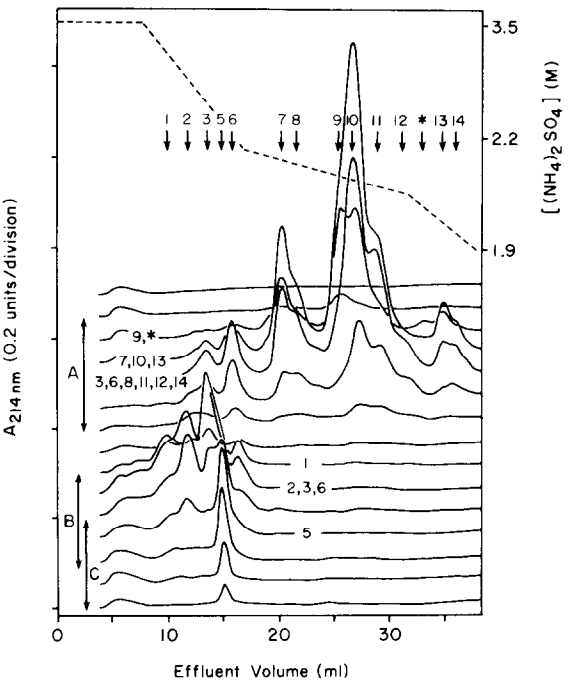

Fig. 7. HIC of fractions obtained by SEC. Approximately 2.2 mg crude *X. laevis* phosvitin/phosvette was chromatographed as in Fig. 2a, but using 0.1 *M* sodium phosphate, pH 6.8, as the eluting buffer with a flow-rate of 0.2 ml/min. Individual 0.7-ml eluant fractions were adjusted to 3.5 *M* $(NH_4)_2SO_4$, applied to a Bio-Gel Phenyl-5-PW column previously equilibrated with 0.1 *M* sodium phosphate, pH 6.8, containing 3.5 *M* $(NH_4)_2SO_4$, and the column eluted with a decreasing concentration of $(NH_4)_2SO_4$ (indicated for the effluent) in 0.1 *M* sodium phosphate, pH 6.8, at a flow-rate of 0.5 ml/min.

TABLE I

CROSS-REFERENCE OF *X. LAEVIS* PHOSVITIN/PHOSVETTE CONSTITUENTS

Each of the components found by SEC is correlated with entities found by other analytical procedures, including gel electrophoresis and the other chromatographic methods described in this report.

SEC (component)	Anion-exchange chromatography (population)	HIC (peak)	Gel electrophoresis (kDa)	Name[a]
A (early)	γ_2	9; 7, 10, 13	31 + 30	$PV_1 + PV_2$
A (late)	γ_1	3,6,8,11,12,14		
B (early)	β	1	13	PVT_2
B (late)		2,3,6		
C	α	5	24	PVT_1

[a] Abbreviations used: PV_1 = phosvitin 1; PV_2 = phosvitin 2; PVT_1 = phosvette 1; PVT_2 = phosvette 2 (for nomenclature, see ref. 7).

SUMMARY AND CONCLUSIONS

A summary of analytical results found for the crude *X. laevis* phosvitin/phosvette fraction is provided in Table I, which cross-references the various phosvitin/phosvette constituents we have observed by a variety of methods.

Previous low pressure SEC of crude *X. laevis* [^{32}P]phosvitin/phosvette on Sephadex G-75 in the presence of 0.5 *M* NaCl indicated two major radioactive and 280-nm absorbing components, designated "peak 1" and "peak 2", with the latter material appearing somewhat heterogeneous [7]. We presume that peak 1 material corresponds to our component A resolved on Superose columns (Fig. 2a), while peak 2 corresponds to our components B + C, even though the relative amounts of 280-nm absorbing material are reversed in the two cases. Low pressure anion-exchange chromatography of these two components on DEAE-cellulose indicated that peak 1 material (= component A) was not further resolved [7], while chromatography on the Mono Q column revealed some heterogeneity in this material (τ-population in Fig. 2b). Peak 2 material, on the other hand, gave rise to two populations by DEAE-cellulose chromatography, designated phosvettes "1" and "2" [7]. Comparable entities reported here are apparently the α- and β-populations, respectively. Both are derived from the B + C couplet (which correspond to peak 2), and the lack of absorbance at 280 nm together with the earlier elution by anion-exchange chromatography identify the α-population as phosvette 1. The earlier elution by anion exchange chromatography further suggests that the α-population (= component C) is either smaller and/or less phosphorylated than the β-population (= component B; phosvette 2); the former possibility, however, is ruled out by gel electrophoretic data (Fig. 4.).

In summary, we have developed rapid procedures to resolve the major phosphoproteins present in *X. laevis* yolk platelets, similar to what had previously been achieved by low pressure chromatography, but within min/h rather than h/days. An efficient plan for the further isolation of each major phosphoprotein would involve the separation of components A from B + C by SEC followed by HIC of components A (to possibly obtain pure populations of phosvitins 1 and 2) and anion-exchange chromatography of components B + C (to maximally resolve phosvettes 1 and 2).

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