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Alkaline Phosphatase Purification: A Comparison of Prep-PAGE with Cyclic Processes

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Abstract

Enzyme purification has been investigated in a preparative-scale polyacrylamide gel electrophoresis system, the Buchler Poly-Prep 200. Experimental results are presented for the optimization of this process using human placental alkaline phosphatase as a typical enzyme. The variables considered are electric field strength, feed concentration, buffer ionic strength, and buffer pH. Enzymes may also be purified by adsorption/desorption onto an ion exchange resin such as DEAE Sepharose in either of two cyclic processes—parametric pumping or cycling zone adsorption. Comparison of the three processes indicates that polyacrylamide gel electrophoresis has the highest purification factor and the greatest enzyme activity recovered, but also the lowest rate of production.

INTRODUCTION

In our recent study of enzyme purification via parametric pumping (*1*), the question was posed as to how this new process compares with conventional separation techniques for enzymes—specifically with regard to purification, enzyme activity recovered, and rate of production. Enzymes are difficult to purify and generally require a series of purification steps (*2–4*). The enzyme, human placental alkaline phosphatase (HPAP), typifies the separation problems encountered in enzyme purification. There are six different forms of the enzyme HPAP, and the crude enzyme mixture contains protein impurities such as human placental albumin with isoelectric points and

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molecular weights close to the values for HPAP, thus making these impurities difficult to remove (*3, 5, 6*).

A typical purification procedure is given by Seargeant and Stinson for human liver alkaline phosphatase (*4*). Their procedure includes butanol extraction and acetone precipitation, followed by ion-exchange and/or affinity chromatography on resins such as DEAE Sephadex, Concanavalin A Sepharose, Phosphonic acid Sepharose, and Sephadex G-200. Ghosh and Fishman (*3*) have proposed continuous paper curtain electrophoresis in addition to Sephadex G-200 gel filtration and DEAE cellulose anion-exchange chromatography as the final steps in a large-scale purification scheme for human placental alkaline phosphatase. The crude enzyme mixture can be easily separated from the placental homogenate by extraction and precipitation or dialysis (*3, 4*). In our previous experiments with HPAP, parametric pumping was investigated as a replacement for the final more-difficult chromatographic steps. In the present work, preparative-scale polyacrylamide gel electrophoresis (prep-PAGE) is proposed as an alternative in the final purification steps.

Parametric pumping is a semicontinuous separation process developed from common chromatographic procedures such as ion-exchange chromatography (*1*) or affinity chromatography (*7*). Semicontinuous processing tends to minimize processing time and, therefore, has the dual advantage of maximizing the rate of production and minimizing the time-dependent degradation of sensitive compounds like enzymes which are generally not very stable.

Parametric pumping is a member of a group of cyclic separation processes, including cycling zone adsorption, which depend on a change in some thermodynamic variable for adsorption and desorption. For purification of HPAP on DEAE Sepharose anion exchanger (*1*), the adsorption/desorption cycle has three major steps involving changes in ionic strength and pH as the intensive variables: (I) adsorption of all proteins at high pH and low ionic strength, pH = 7.4 and I.S. = 0.1 *M*; (II) selective desorption of the desired enzyme at high pH and high ionic strength, pH = 7.4 and I.S. = 0.6 *M*; and (III) desorption of protein impurities at low pH and low ionic strength, pH = 4.0 and I.S. = 0.1 *M*. In parametric pumping, an oscillating direction of fluid flow is coupled to the change in the thermodynamic variable. The oscillating fluid flow tends to amplify the concentration waves giving better separation or purification than one-way processes. Cycling zone adsorption is similar to parametric pumping except that the flow is unidirectional. Chen et al. (*1*) compared parametric pumping and cycling zone adsorption for the purification of HPAP and showed that the former process yields a larger percentage of the total enzyme activity recovered and greater purification

while the latter process yields a higher rate of production. These processes are compared with prep-PAGE below.

We have recently demonstrated that prep-PAGE can be operated in a semicontinuous manner, i.e., cycling zone electrophoresis, for protein separations (*8*). PAGE has previously been used for analytical separations of HPAP samples (*9, 10*). The disk gel patterns show excellent resolution, thus indicating a strong potential for the use of cycling zone prep-PAGE in enzyme purification.

Polyacrylamide gel was introduced as a new stabilizing medium for electrophoresis by two independent groups of researchers in 1959, Raymond and Weintraub (*11*) and Davis and Ornstein (*12*). The sharpness of the individual protein bands and the number of separate bands observed were reported to be superior compared to preexisting electrophoretic procedures such as starch gel, agar gel, or paper electrophoresis. Reviews of the development of the procedures and equipment for both analytical-scale and preparative-scale PAGE are available in the literature (*13–16*).

A Buchler Poly-Prep 200 preparative-scale electrophoresis system was used in the current investigation. This apparatus is based on the design by Chrambach and co-workers (*13, 17*). Note that the term "preparative-scale" implies load capacities in the order of 1 mg/cm^2 of cross-sectional area of gel. Righetti and Secchi (*18*) have used prep-PAGE for the separation of protein mixtures in a LKB UNIPHOR 7900 electrophoresis system. Because of the limited capacity of this milligram-scale equipment, prep-PAGE is only recommended as one of the final process steps after the quantity of starting material has been considerably reduced.

Righetti and Secchi (*18*) studied the optimal conditions for the separation of proteins such as bovine serum albumin, ovalbumin, lysozyme, and cytochrome C via prep-PAGE. They recommended gel strengths ranging from 4 to 8% acrylamide, gel heights of 5 to 10 cm, elution buffer rates of 12 to 25 mL/h, and buffer ionic strengths of 0.02 to 0.03 *M*. An optimization problem occurs in electrophoretic separations with respect to the length of time required for the individual proteins to move through the gel. If the product elution time is too short, successive concentration peaks will not be completely developed, resulting in poor separation. On the other hand, if the time period is too long, axial diffusion will tend to broaden the individual peaks leading to overlapping of successive bands and to poor separation. In order to maximize the separation, the variables such as gel height, buffer ionic strength, buffer pH, and electric field strength which control the total time for protein movement through the gel must be optimized. The optimal conditions are dependent on the nature of the components of each mixture to be separated, and must be determined experimentally.

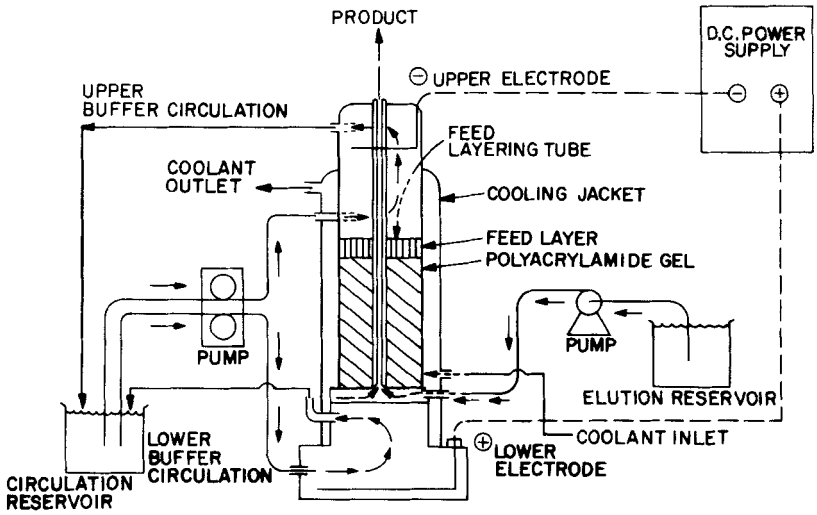

FIG. 1. Experimental apparatus.

EXPERIMENTAL

HPAP with a specific activity of 3.0 units/mg of solid was purchased from Sigma Biochemicals. By definition, one unit of enzyme activity will hydrolyze 1 μmol of *p*-nitrophenyl phosphate per minute at 303 K and at a pH of 10.15 (*6*). The activity of pure HPAP is approximately 1171 units/mg (*4*). The detailed composition of the impurities in the Sigma HPAP is not known, but the major impurity is probably human placental albumin (*4*). The electrophoretic elution profiles for the major impurity in the experiments below closely match the elution profiles for human serum albumin under identical operating conditions.

The experimental set-up is shown in Fig. 1. The electrophoresis system includes the Buchler Poly-Prep 200 apparatus, two Buchler multistaltic pumps, and a Buchler 3-1500 direct current power supply. One multistaltic pump was used to circulate the buffer solution through the cathode and anode compartments to an external buffer reservoir (1-L volume). The buffer was titrated in the external reservoir to maintain the desired pH. A second multistaltic pump was used to pump precooled elution buffer into the elution chamber and up through the central elution capillary tube to the product stream. The Buchler Poly-Prep system was maintained at 278 K by circulation of cooling water through the inner and outer cooling jackets.

The Buchler Poly-Prep apparatus has two main parts. The upper section contains the electrophoresis column, the upper electrode chamber, the central elution capillary, and the inner and outer cooling jackets. The lower section contains the lower electrode chamber and the elution chamber, separated by a semipermeable glass membrane. Prior to the start of each experiment, the polyacrylamide gel is polymerized directly in the prep-PAGE system. When the two sections of the Buchler Poly-Prep apparatus are assembled, the bottom of the self-supporting gel forms the upper boundary of the elution chamber. The gel has an annular cross-sectional area of 18 cm^2. A gel height of 8.3 cm was used for all of the experimental runs.

The gel must be meticulously prepared in order to obtain reproducible data. The composition of the gel was 5 wt% acrylamide, 0.13 wt% *N,N'*-methylene bis acrylamide, 0.15 wt% ammonium persulfate, plus 0.07 vol% TEMED (*19*). The gel solution was titrated to a pH of 8.6 with 0.10 *M* Tris buffer solution, then filtered and poured slowly into the column, being careful to avoid formation of air bubbles. A thin layer of distilled water was gently layered onto the surface of the gel solution in order to prevent the formation of a miniscus on top of the gel during polymerization. After allowing the gel to polymerize completely (~30 min), the surface of the gel was rinsed with fresh water and the Buchler Poly-Prep apparatus was assembled and filled with buffer solution. The power supply was set at constant voltage (200 V), and the system was run for 6 h to remove ultraviolet detectable materials from the gel.

The buffer was prepared by mixing solutions of Tris and glycine of the same molarities. In the experiments below, the buffer ionic strength was varied from 0.05 to 0.20 *M* and the pH was varied from 8.0 to 9.5 as shown in Table 1. The feed solution was prepared by adding 0.02 to 0.05 wt% Sigma HPAP and 10.0 wt% sucrose to the Tris/glycine buffer solution. The added sucrose makes the feed solution denser than the buffer solution. The feed is pumped into the upper compartment through the feed layering tube. The feed solution forms a distinct layer directly above the gel and directly below the circulating buffer solution. The dense feed layer remains in place throughout an entire experimental run. We have previously shown that the gel can be reused a number of times (*8*). In cycling zone electrophoresis, the spent feed is periodically removed and fresh feed is pumped into the system.

Product fractions with volumes of 5 mL each were collected at a product elution rate of 20 mL/h. These samples were analyzed using a Bausch and Lomb spectrophotometer. Bio-Rad protein assay was used to determine total protein concentration at a wavelength of 595 nm and 293 K. The enzyme activity of the product was determined by measuring the increase in

TABLE 1
Experimental Conditions and Results

Run	Volts	Current (mA)	Watts	I.S. (*M*)	pH	Feed (wt%)	P.F.	ψ (%)
			Constant Power Runs					
1	140	14	2	0.05	8.6	0.02	2.04	98
2	250	20	5	0.05	8.6	0.02	3.25	98
3	277	22	6	0.05	8.6	0.02	2.54	95
4	312	26	8	0.05	8.6	0.02	2.07	69
5	360	28	10	0.05	8.6	0.02	1.86	37
6	203	25	5	0.10	8.6	0.02	3.32	94
7	180	28	5	0.20	8.6	0.02	3.42	97
			Constant Voltage Runs					
8	250	28	7	0.10	8.6	0.02	3.26	93
9	250	36	9	0.20	8.6	0.02	3.49	89
10	250	18	4.5	0.10	8.0	0.02	2.54	89
11	250	28	7.0	0.10	9.0	0.02	2.92	86
12	250	19	4.8	0.10	9.5	0.02	2.49	88
13	250	34	8.5	0.10	8.6	0.05	2.03	92

absorbance after reaction with *p*-nitrophenyl phosphate at 405 nm and 303 K as described in the *Worthington Enzyme Manual* (*6*).

The experimental set-ups are similar for parametric pumping and cycling zone adsorption. A jacketed ion-exchange column replaces the electrophoresis column in the center of Fig. 1. A Pharmacia column (1.6 cm i.d.) was packed with DEAE Sepharose, an anion exchanger, to a bed height of 8.0 cm. Cooling water was circulated through the jacket of the column in order to maintain the system at 278 K.

Two reversible peristaltic pumps (manufactured by Pharmacia Fine Chemicals) replace the two Buchler multistaltic pumps in Fig. 1. One peristaltic pump was used to alternately introduce the HPAP feed solution (pH = 7.4 and I.S. = 0.1 *M*), the high pH elution buffer (pH = 7.4 and I.S. = 0.6 *M*), and the low pH elution buffer (pH = 4.0 and I.S. = 0.1 *M*) to the top or bottom of the ion-exchange column. Dialyzers were used to maintain the I.S.'s at the desired values, and the second peristaltic pump was used to circulate the appropriate buffer solutions through the shell-side of the dialyzers. More external reservoirs are used for the cyclic processes than for the electrophoretic process (complete details are given in Ref. 1). The solutions in the various reservoirs were titrated to maintain the desired pH's.

The high pH buffer for the cyclic processes was prepared by mixing solutions of the appropriate molarity of Tris and HCl, and the low pH buffer was a mixture of acetic acid and sodium acetate. The HPAP starting material and the analytical procedures were identical to the present work.

RESULTS AND DISCUSSION

The experimental operating conditions for 13 runs on HPAP are listed in Table 1. Tris/glycine buffer was used for all of the experiments. Rodbard and Chrambach suggest using different buffer components for the circulating buffers in the upper and lower electrode chambers and also for the gel preparation (*19*). The operation of the prep-PAGE system is simplified in the present work by the use of only one buffer solution.

The power settings in the various experiments vary from 2 to 10 W. An experiment was attempted at 20 W, but serious degradation of the gel occurred due to overheating. The electric power generates heat and the cooling capacity of the Buchler Poly-Prep system is mechanically limited.

Ahmed et al. studied paper electrophoresis of HPAP using veronal, borate, and phosphate buffers and achieved the best resolution with phosphate buffer (*20*). In the present work, preliminary runs were made with phosphate buffer (NaH_2PO_4 plus Na_2HPO_4), Tris-maleate plus NaOH buffer, and Tris plus glycine buffer. The migration velocities of the various proteins are directly proportional to the strength of the applied electric field, and higher voltages were obtained at a given wattage with the Tris/glycine buffer. At 10 W constant power, the voltage was approximately 120 V in 0.05 *M* phosphate buffer, 210 V in 0.05 *M* Tris-maleate/NaOH buffer, and 360 V in 0.05 *M* Tris/glycine buffer. The Tris/glycine buffer carries less current and thus generates less heat at the higher voltage because it consists of two organic components which are only partially ionized.

A typical experimental run is shown in Fig. 2. The ratio of the concentration y_i of total protein in each product fraction to the feed concentration y_F is plotted at the top of the figure. Two major peaks were resolved. By comparison with the activities plotted at the bottom of the figure, it is seen that the protein in the first peak has nearly zero enzyme activity a_i, where a_i is expressed in international units per volume of sample. The protein in the second peak has a high enzyme activity. The second peak is, therefore, identified as the desired enzyme and the first peak is identified as the major impurity. Other minor components of the crude enzyme mixture appear as minor peaks on the shoulders of the two major peaks.

The protein with high activity is taken as the product and is indicated by the hatched areas in Fig. 2. The purification factor P.F. is defined as the ratio

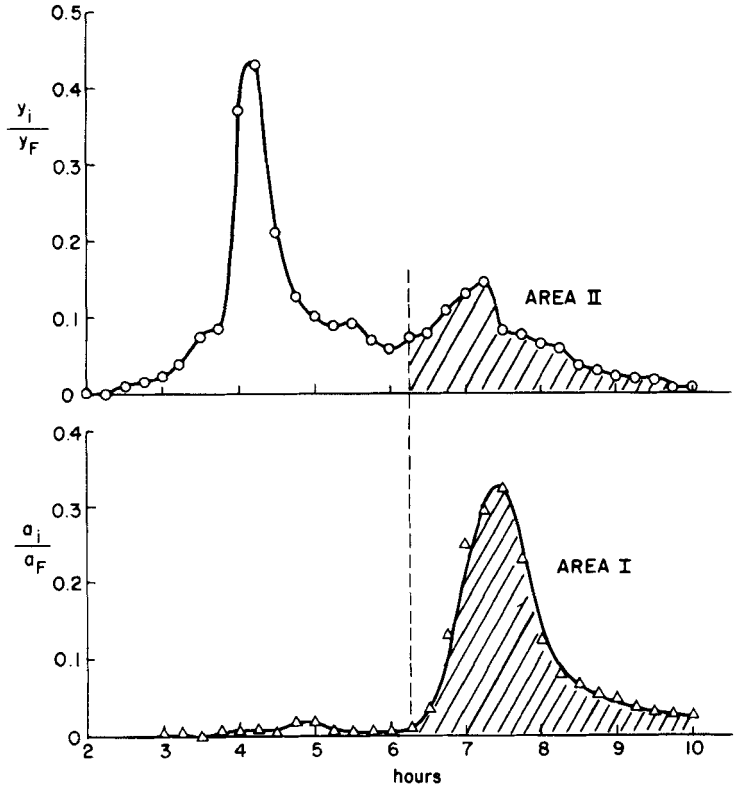

FIG. 2. Determination of purification factor (Run 2).

of the activity per milligram of product to the activity per milligram of feed or the ratio of the hatched areas in Fig. 2.

$$\text{P.F.} = \frac{\Sigma a_i v_i / \Sigma y_i v_i}{a_F / y_F} = \frac{\text{Area I}}{\text{Area II}} \tag{1}$$

The percent enzyme activity recovered ψ is the ratio of the total enzyme activity in the product to the total enzyme activity in the feed.

$$\psi = \frac{\Sigma a_i v_i}{a_F F} \times 100\% \tag{2}$$

The purification factors vary from 1.9 to 3.5 and the recoveries vary from 37 to 98% in the experiments in Table 1.

In previous experiments with human hemoglobin and human serum albumin in the Buchler Poly-Prep system, better separation was obtained for a feed volume of 10 cc than for a feed volume of 30 cc (*8*). Figure 3 compares the separation obtained for 10 cc of 0.05 wt% HPAP feed (Run 13) versus 10 cc of 0.02 wt% HPAP feed (Run 8). The enzyme activity peaks are identical in both runs, but the lower feed concentration in Run 8 gives a much sharper peak for the impurity and, therefore, a significant improvement in purification. A 10-cc volume of 0.02 wt% HPAP feed was used in the remaining experiments.

Righetti and Secchi give a value for the maximum sample load in prep-PAGE of 10 mg of protein per cm^2 per protein band, and recommend that the

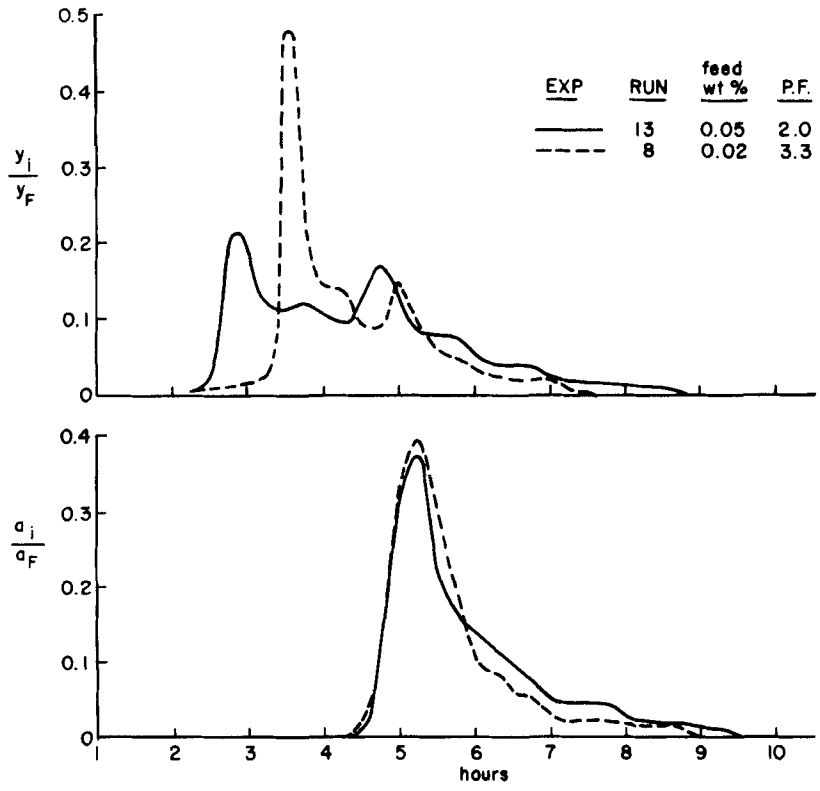

FIG. 3. Effect of feed concentration on elution profiles.

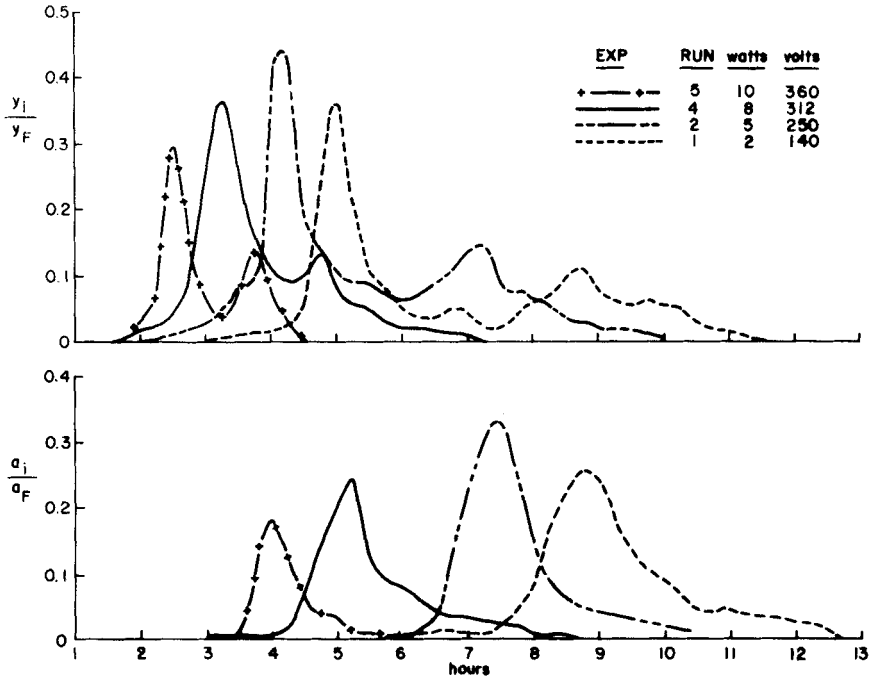

FIG. 4. Effect of power on elution profiles.

load be reduced to 2 mg of protein per cm^2 per protein band for mixtures whose components give closely adjacent bands (*18*). The much smaller loading in the present work of 0.056 mg of crude enzyme per cm^2 per protein band is necessitated by the difficulty of separating HPAP from the accompanying impurities.

The effect of increased power and voltage on the separation is studied in Fig. 4. As the power and voltage increase, both the impurity and the enzyme move at a faster rate through the gel and exit the prep-PAGE system in a shorter time. The sharpest peaks for the concentrations of the impurity and the enzyme and also for the activity of the enzyme occur at 5 W or 250 V (Run 2). As seen in Fig. 5, the purification factor is maximized at 5 W. At higher wattages, the peaks are too close together giving poor separation. At lower values than 5 W, the residence time in the system is too long, giving broad diffuse peaks and poor separation.

The recovery as a function of power is also plotted in Fig. 5. The recovery is excellent up to 6 W but drops off drastically at 8 to 10 W. As seen in the

bottom of Fig. 4, the area under the enzyme activity peak is much smaller at 10 W than at lower fields. In our previous experiments with human hemoglobin and human serum albumin, the separation also went through an experimental optimum around 5 W but the recovery was good up to 10 W (*8*).

The effect of pH on purification and recovery is examined in Figs. 6 and 7. HPAP has an isoelectric point I_A of 4.6 (*21*), and the isoelectric point of the major impurity is apparently in the same range. As $|\text{pH} - I_A|$ increases, the net charge carried by both the protein impurity and the enzyme molecule increases, thus increasing the migration velocities of both components of the crude enzyme mixture in the Buchler Poly-Prep system. As seen in Fig. 6, the concentration and activity peaks move at a faster rate at higher pH's as expected. The enzyme activity peak is approximately the same shape at the various pH values. The concentration peaks for the enzyme and the impurity are sharper at the higher pH values, but are also slightly closer together. The best purification is obtained at an operating pH of about 8.6 (Fig. 7). The recoveries are approximately constant in the pH range of 8.0 to 9.5. Ahmed

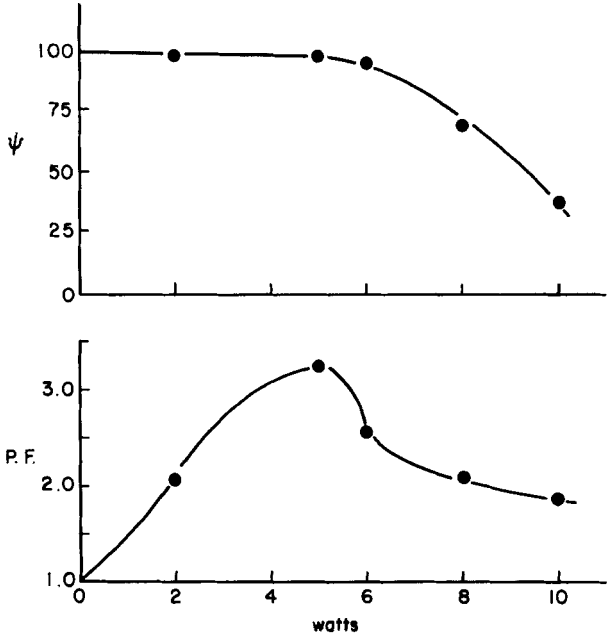

FIG. 5. Optimization of enzyme activity recovered (top) and purification factor (bottom) with respect to power.

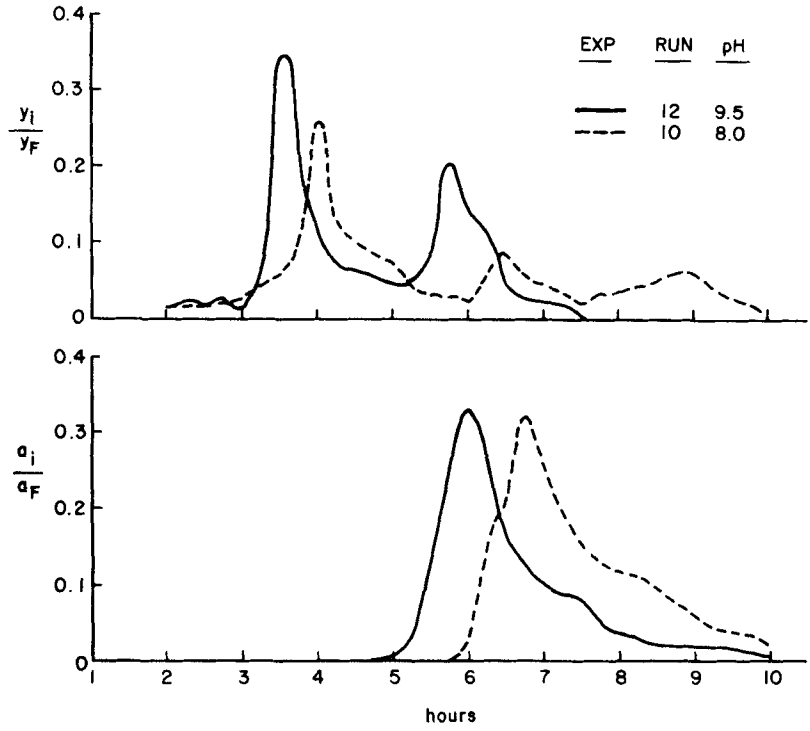

FIG. 6. Effect of buffer pH on elution profiles.

et al. reported the best separations in paper electrophoresis of various types of alkaline phosphatase at pH values of 8.0 to 8.6, some separation at pH values of 7.2, and poor separation at pH values of 4.6 to 6.8 (*20*). Preliminary experiments in the Buchler Poly-Prep system gave very poor results at pH 7.2.

The effect of buffer ionic strength on the purification and recovery of HPAP is shown in Figs. 8 and 9. The electrophoretic elution curves in Fig. 8 show very little change when the ionic strength is increased at constant power. Since the 0.20 *M* buffer carries more current at 5 W than the 0.05 *M* buffer, the voltage is decreased and the peaks are slightly delayed in Run 7 as compared with Run 2. For the two runs at constant voltage (Run 2 versus Run 9), the concentration and activity peaks exit the prep-PAGE system much earlier at the higher buffer molarity—the reason for this phenomenon is not clear.

The purification factor increases very slightly as the ionic strength increases from 0.05 to 0.20 *M* as shown in Fig. 9. At 5 W constant power the recovery is approximately constant. At 250 V constant field, however, the power along with the inherent heating effects increase as the buffer molarity increases, causing a decrease in recovery.

The recoveries and purification factors are summarized in Table 1. The best results were obtained in Runs 2 and 7 with purification factors of 3.3 to 3.4 and enzyme recoveries of 97 to 98%. Table 2 compares these results with the results obtained by Chen et al. for parametric pumping and cycling-zone adsorption (*1*). The purification and recovery for prep-PAGE are superior to the values obtained for the cyclic processes, but the rate of production is quite low. DEAE Sepharose anion exchanger was used in the cyclic processes, so the results in Table 2 are relevant to replacement of one of the chromatographic steps in a typical enzyme purification scheme.

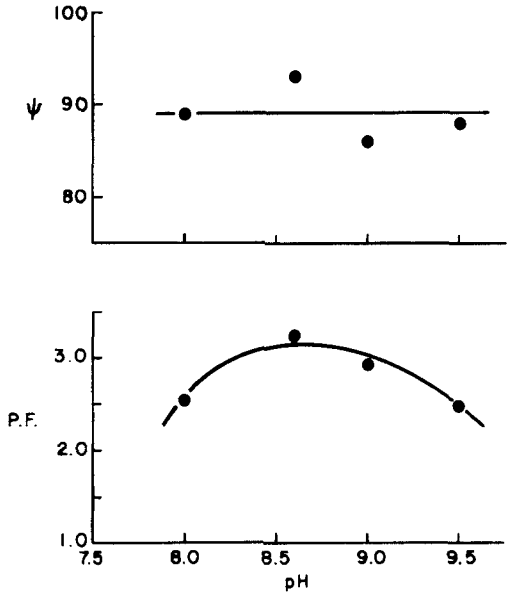

FIG. 7. Optimization of enzyme activity recovered (top) and purification factor (bottom) with respect to buffer pH.

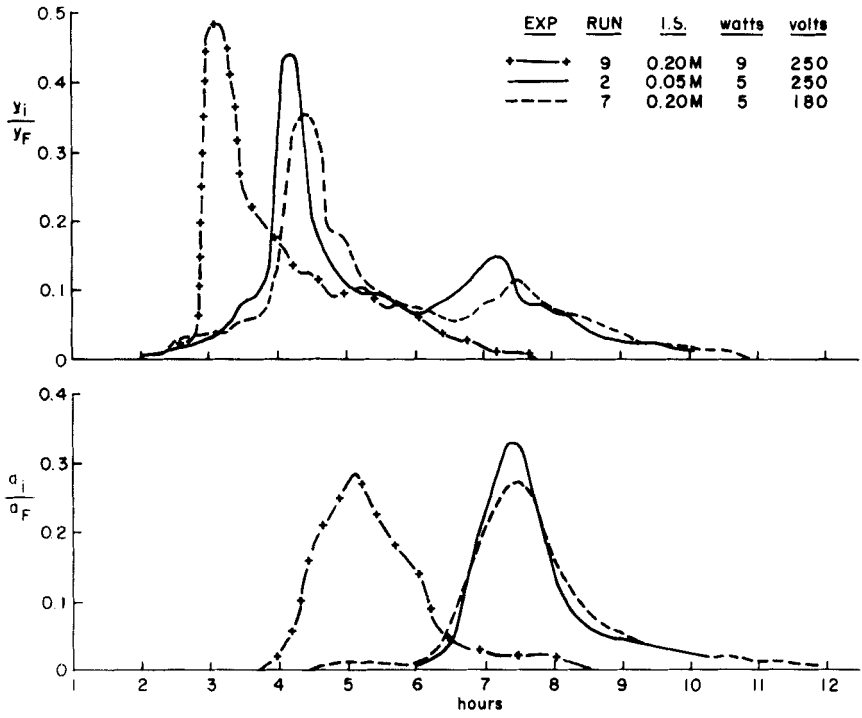

FIG. 8. Effect of buffer ionic strength on elution profiles.

CONCLUSIONS

Prep-PAGE has been demonstrated to be a feasible process step for enzyme purification. Optimum results were obtained at 5 W power, at a buffer pH of 8.6, and at a feed loading of 10 cc of 0.02 wt% crude enzyme. A gel strength of 5 wt% acrylamide, a gel height of 8.3 cm, and an elution buffer rate of 20 mL/h were used for all of the experiments. The buffer ionic strength had little effect on the experimental results over the range of 0.05 to 0.20 *M* using a Tris/glycine buffer solution. The experimental results obtained via prep-PAGE are superior to the results achieved via DEAE-type anion exchangers for the purification and recovery of HPAP.

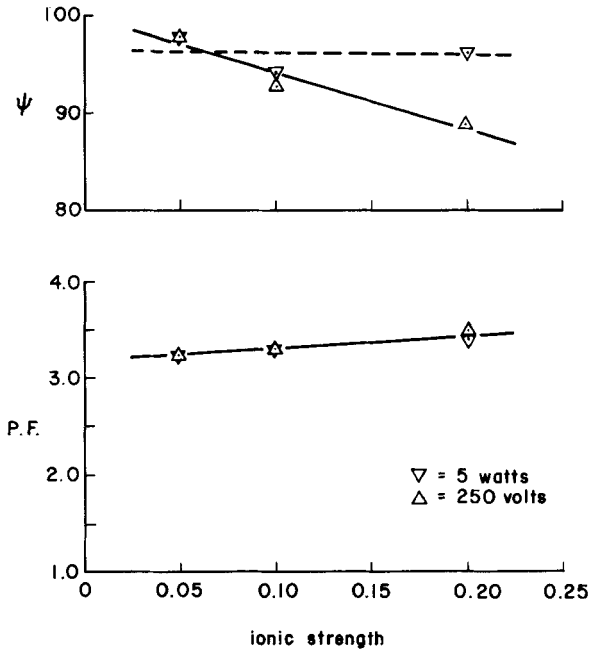

FIG. 9. Optimization of enzyme activity recovered (top) and purification factor (bottom) with respect to buffer ionic strength.

TABLE 2
Comparison of Prep-PAGE with Cyclic Processes

	Prep-PAGE	Parametric[a] pumping	Cycling zone[a] adsorption
Purification factor, P.F.	3.4	2.8	1.6
% Enzyme activity recovered, ψ	97	75	59
Rate of production, international units per cm^2 per hour	0.027	1.9	3.6

[a]Data from Ref. *1*.

SYMBOLS

a_i	activity of product sample (international units/mL)
a_F	activity of feed solution (international units/mL)
F	volume of feed (mL)
HPAP	human placental alkaline phosphatase
I.S.	ionic strength or molarity of buffer solution
P.F.	purification factor defined by Eq. (1)
prep-PAGE	preparative-scale polyacrylamide gel electrophoresis
v_i	volume of product sample (mL)
y_i	concentration of product sample (mg/mL)
y_F	concentration of feed (mg/mL)

Greek Letters

ψ	% enzyme activity recovered

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