New Procedure for Purifying and Crystallizing Alkaline Phosphatase from Human Intestinal Mucosa

Sheshadri Narayanan and Harold D. Appleton

A new procedure is described for extracting, purifying, and crystallizing alkaline phosphatase orthophosphoric monoester phosphohydrolase (EC 3.1.3.1) from human intestinal mucosa. Active enzyme, extracted with trichlorotrifluoroethane, is subsequently purified by chromatography on Sephadex G-200 and DEAE Sephadex. The acetone and ammonium sulfate precipitation steps that are part of the conventional butanol extraction and purification procedure are eliminated. Enzyme activity is concentrated at each stage of the purification procedure, yielding a preparation with a specific activity three times greater than that obtained with the conventional butanol procedure.

Additional Keyphrases  trichlorotrifluoroethane extraction •  Sephadex-gel permeation •  anion-exchange chromatography •  immunodiffusion •  chromatography on DEAE Sephadex

The classic procedure for extracting alkaline phosphatase (orthophosphoric monoester hydrolase, EC 3.1.3.1) activity from animal tissues with butanol was first introduced by Morton (1).

This procedure, as modified by Moss et al. (2), entailed extracting the enzyme with butanol, fractionation with acetone and ammonium sulfate, followed by Sephadex-gel permeation and anion-exchange chromatography. This resulted in a low yield of a highly purified preparation (2). This five-step purification procedure is time-consuming, and the low yields obtained have been attributed to ammonium sulfate coordinating with zinc in the active center of the enzyme (3).

The procedure described in this paper involves only three steps: extraction of the enzyme activity with trichlorotrifluoroethane ("Genetron-113") followed by purification by use of Sephadex-gel permeation and anion-exchange chromatography.

Materials and Methods

Materials

Trichlorotrifluoroethane (Genetron-113; Allied Chemical Co., Morristown, N. J. 07960). Sephadex G-200 and DEAE (diethylaminoethyl) Sephadex (Sephadex A-50) were products of Pharmacia Fine Chemicals, Inc., Piscataway, N. J. 08854. Powdered "Carbowax" (polyethylene glycol 6000) was a product of Carbide and Carbon Chemicals Co., New York, N. Y. 10017. Tris(hydroxymethyl)-aminomethane hydrochloride (Tris HCl), and ammonium sulfate, both specified as "ultra pure," and the disodium salt of p-nitrophenylphosphate were products of Schwarz/Mann, Orangeburg, N. Y. 10962. n-Butanol was a product of J. T. Baker Chemical Co., Phillipsburg, N. J. 08865.

Apparatus

Columns and accessories for gel permeation and anion-exchange chromatography were purchased (Pharmacia Fine Chemicals, Inc.).

The gradient elution apparatus we used for anion-exchange chromatography was purchased...
from Glass Engineering Co., Houston, Texas 77009.

Eluent fractions from Sephadex-gel permeation and anion-exchange chromatography were collected in tubes mounted on a Rinco automatic fraction collector (GM Instrument Co., Greenville, Ill. 62246). The polystaltic pump used to siphon sample and buffer into packed chromatographic columns was manufactured by Buchler Instrument Co., Fort Lee, N. J. 07024.

Cellulose acetate electrophoresis was performed on apparatus manufactured by Gelman Instrument Co., Ann Arbor, Mich. 48106, with which their Sephaphore III cellulose polyacetate strips were used.

Protein elution profiles of effluents from Sephadex-gel permeation and anion-exchange chromatographic columns were monitored at 280 nm with a Model DB Spectrophotometer with a 1-cm lightpath cuvet (Beckman Instruments, Inc., Fullerton, Calif. 92634)

All other spectrophotometric measurements were made on a “Spectronic 20” spectrophotometer (Bausch & Lomb, Rochester, N. Y. 12643).

Immunodiffusion plates (ID 7 cell) were purchased from Cordis Corp., Miami, Fla. 33137.

Procedures

Preparation of human intestinal mucosal homogenate. Specimens of small intestine, the whole length from the duodenum to the ileum, were collected at autopsies performed within 24 hours of death. The convolutions of the intestine were removed by cutting away the mesenteric tissue, and the remaining specimen was rinsed with tap water. After fatty tissue was removed, the small intestine was frozen until ready for use.

Before preparation of the homogenate, the small intestine was thawed just enough to allow it to be laid flat on a wooden board, and the still firm mucosa was scraped off with a scalpel.

The mucosal scrapings were then homogenized with sufficient Tris buffer (10 mmol/liter, pH 7.6) in a Waring Blender at high speed for about 2 min. The volume of buffer used per gram of mucosa was 2.6 to 3 ml. Alkaline phosphatase was released from the homogenate into the aqueous phase by using either 1-butanol or trichlorotrifuoroethane (Genetron-113).

Butanol extraction and purification procedure. Essentially, Morton's butanol extraction procedure (1) as modified by Moss et al. (2) was used. This entailed release of the enzyme from the intestinal mucosal homogenate into the aqueous phase, fractionation with acetone and ammonium sulfate, and finally purification by use of Sephadex-gel permeation and anion-exchange chromatography.

Genetron extraction and purification procedure. Equal volumes of human intestinal mucosal homogenate and Genetron-113 were mixed in a vortex-type mixer at high speed for 2 min. The homogenized mixture separated into three phases on standing: a top aqueous phase, containing the enzyme activity, a middle gel-like phase containing denatured protein, and a lower solvent phase. Even though these separations appeared clear-cut, the mixture was centrifuged at 10,000 revolutions per minute (12,100 × g) for 10 min at 25°C in a Servall refrigerated centrifuge to ensure complete separation of the phases. The aqueous (top) layer was carefully removed by siphoning. The remaining phases were submitted to three more extractions with equal volumes of Genetron-113 and Tris buffer (10 mmol/liter, pH 7.6), the mixtures centrifuged as before, and the aqueous phases collected. The aqueous phases resulting from the four Genetron extractions were pooled, and concentrated by dialysis against Carbowax overnight in the cold room. The dialysate, referred to as the Genetron extract, was purified by use of Sephadex G-200, and Sephadex A-50 (DEAE Sephadex) chromatography.

Sephadex-gel permeation chromatography of the Genetron extract. Concentrated Genetron extract was pumped upwards through a 2.5 × 90 cm laboratory column (Pharmacia) packed with Sephadex G-200 and fitted with flow adapters. The eluting buffer was Tris-HCl (10 mmol/liter, pH 7.6) and contained 0.1 mol of sodium chloride per liter. Fractions containing the enzyme activity were pooled and concentrated against Carbowax, and this concentrated extract was subjected to anion-exchange chromatography.

Sephadex A-50 (DEAE Sephadex) chromatography. The concentrated extract resulting from Sephadex G-200 chromatography was pumped downward through a 2.5 × 45 cm laboratory column (Pharmacia) packed with Sephadex A-50 (DEAE Sephadex). After applying the sample, we connected the column to a gradient elution apparatus consisting of a mixing flask and a reservoir flask. The reservoir flask held 250 ml of Tris-HCl buffer (10 mmol/liter, pH 7.7) containing sodium chloride (0.3 mol/liter), and was connected to the mixing flask, which contained 250 ml of Tris-HCl (10 mmol/liter, pH 7.7). The mixing flask was continually stirred with a magnetic stirrer. With this gradient elution, the fractions contributing to the major peak of enzyme activity were pooled and concentrated against Carbowax.

Electrophoresis. Cellulose-acetate electrophoresis of the crude Genetron extract, and of the preparation resulting from Sephadex A-50 chromatography, was performed (barbital buffer, pH 8.6, ionic strength 0.025). At the cathodic supporting bridge, 25 μl of sample was applied and run at
250 V for 20 min. Some of the strips were stained for protein; others were stained to locate enzyme activity. Ponceau S dye (0.5 g/100 ml of trichloroacetic acid, 7.5 g/100 ml) was used as a protein stain. A diazo dye, Fast Blue RR (0.2 g/100 ml), sodium α-naphthyl acid phosphate (1 g/100 ml), and Tris-HCl buffer (0.1 mol/liter, pH 8.6) was the stain used to localize enzyme activity (4).

Crystallization of the enzyme. An aliquot of the concentrated extract resulting from Sephadex A-50 chromatography (after dialysis against Carbowax) was diluted with an equal volume of Tris-HCl buffer (10 mmol/liter, pH 7.4, ionic strength 0.008), and stored at 4°C. Crystals appeared after about four weeks.

Preparation of antibody and immuno-gel diffusion experiments. Purified enzyme [a solution of crystalline enzyme in Tris-HCl (10 mmol/liter, pH 7.4) with an activity of 33 U/ml] was emulsified with Freund's adjuvant (5, 6) before it was injected into rabbits. The rabbits were bled from the marginal ear vein six weeks after the first immunization. An aliquot of the antiserum was reacted with three volumes of "Rivanol" (2-ethoxy-6,9-diamino-acridine lactate), 0.4 g/100 ml of Tris-HCl buffer (10 mmol/liter, pH 7.6), to precipitate all non-gamma globulin proteins. The mixture was centrifuged and the supernatant fluid clarified by passage through acid-washed Norit charcoal. The clear solution was concentrated by dialysis against Carbowax, and the concentrated antiserum was placed in the central well of the immuno-gel diffusion plate. The peripheral wells contained undiluted and serially diluted enzyme (antigen) in saline.

Analytical assays. Units of enzyme activity reported in this paper represent μmoles of p-nitrophenol released per minute per milliliter of the enzyme solution at 25°C. The release of p-nitrophenol from 1 millimolar p-nitrophenylphosphate in Tris buffer (0.1 mol/liter, pH 7.8) was measured by its absorbance at 410 nm (7). (Because one of the reasons for this investigation was to later perform kinetic and other studies with the purified enzyme near the physiological pH value, pH 7.4, enzyme activity at the various stages of purification was not assayed at its pH optimum, which is near pH 10 for p-nitrophenylphosphate.)

Protein content at each stage of the purification procedure was determined at 750 nm with Folin–Ciocalteau reagent (8).

Results

Table 1 summarizes the results obtained at each stage of the butanol extraction procedure. The starting material was 78 g (wet weight) of human intestinal mucosa, which was homogenized in 200 ml of Tris-HCl (10 mmol/liter, pH 7.6). Of this homogenate, 100 ml was extracted with butanol as discussed before. The remaining 100 ml was extracted with Genetron-113.

The specific activity (units of enzyme activity per milligram of protein) of the enzyme preparation at the "butanol extract" stage of purification was 0.085. At the "acetone precipitate" stage of purification the specific activity increased to 0.317. There was no appreciable increase in specific activity at the "ammonium sulfate" stage of purification. However, the specific activity rose from 0.96 at the "ammonium sulfate" stage of purification to 19.0 after Sephadex-gel permeation chromatography. This degree of purity was achieved at the expense of an appreciable loss in enzyme activity. Thus, the Sephadex G-200 extract had an enzyme activity of 13 units/ml as compared with the preparation at the "ammonium-sulfate" stage of purification, when the activity was 50 U/ml. The Sephadex G-200 gel permeation chromatography also resulted in freeing the enzyme activity from a considerable amount of extraneous protein. Thus the Sephadex G-200 enzyme extract had only 0.7 mg of protein per milliliter, as compared with the 52 mg of protein per milliliter at the previous step.

The results in Table 1 show that Sephadex A-50 chromatography did not improve the purity of the preparation obtained after Sephadex G-200 chromatography.

Table 2 summarizes the results obtained in the three-step Genetron extraction and purification procedure. The specific activity of the enzyme preparation at the "Genetron extract" stage of purification was 0.354. This was in contrast to the specific activity of 0.085 obtained at the "butanol extract" stage of the butanol extraction and purification procedure. On Sephadex G-200 gel

<table>
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<tr>
<th>Table 1. Purification of Alkaline Phosphatase Activity from Human Intestinal Mucosa by the Butanol Extraction Procedure</th>
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<tbody>
<tr>
<td>Stage of purification</td>
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<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Butanol extract</td>
</tr>
<tr>
<td>50% (v/v) acetone</td>
</tr>
<tr>
<td>precipitate</td>
</tr>
<tr>
<td>ammonium sulfate</td>
</tr>
<tr>
<td>Sephadex G-200</td>
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<td>Sephadex A-50</td>
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permeation chromatography of the Genetron extract the specific activity of the enzyme preparation rose to 63.0. Sephadex G-200 gel permeation chromatography also freed the enzyme preparation from much extraneous protein without seriously affecting the activity of the enzyme: the Sephadex G-200 purified Genetron extract had only 0.7 mg of protein per milliliter as compared with the 144 mg of protein per milliliter of the initial Genetron extract.

Enzyme activity and protein elution profiles obtained on Sephadex G-200 gel-permeation chromatography of the Genetron extract are shown in Figure 1. The enzyme elution profile depicted in Figure 1 indicates homogeneity.

While the specific activity at the Sephadex G-200 gel permeation chromatography stage of the Genetron extract was 63.0, the specific activity after Sephadex G-200 chromatography of the butanol extract was only 19.0.

As in the butanol extraction and purification procedure, Sephadex A-50 chromatography did not improve upon the purity of the Sephadex G-200 purified "Genetron extract."

Figure 2 depicts the protein and enzyme elution profiles obtained on Sephadex A-50 chromatography of the Sephadex G-200 purified Genetron extract. A slight heterogeneity is indicated in the enzyme elution profile depicted in Figure 2. There are two minor peaks, followed by one broad, major, and nearly homogenous peak of enzyme activity. Only the fractions corresponding to the major peak were pooled and concentrated by dialysis against Carbowax.

Figure 3 compares the electrophoretic profile obtained on cellulose-acetate electrophoresis of the crude Genetron extract with the profile obtained on electrophoresis of the enzyme after purification on Sephadex A-50. There are two minor bands and a major band in the electrophoreto-

<table>
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<tr>
<th>Stage of purification</th>
<th>Total volume (ml) concentrated against Carbowax</th>
<th>Enzyme activity, U/ml</th>
<th>Protein, mg/ml</th>
<th>III/IV, specific activity, units/mg of protein</th>
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<tr>
<td>Genetron extract</td>
<td>6.5</td>
<td>51.0</td>
<td>144</td>
<td>0.354</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>3.5</td>
<td>44.0</td>
<td>0.70</td>
<td>63.0</td>
</tr>
<tr>
<td>Sephadex A-50</td>
<td>3.5</td>
<td>32.4</td>
<td>0.60</td>
<td>54.0</td>
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</table>

Fig. 1. Sephadex G-200 gel permeation chromatography of the "Genetron extract" of homogenate of human intestinal mucosa

Eluting buffer, Tris-HCl (0.01 mol/liter, pH 7.6), containing 0.1 mol of sodium chloride per liter. Void volume, 135 ml (no enzyme activity in the void volume). Effluent flow rate, 32 ml/h. 8-ml fractions collected. "Abs" is the protein elution profile (absorbance of the fractions at 280 nm)

Fig. 2. Sephadex A-50 (DEAE Sephadex) chromatography of the Sephadex G-200 purified "Genetron extract"

Gradient eluted, with 250 ml of Tris-HCl (0.01 mol/liter, pH 7.7) in the mixing flask, and 250 ml of Tris-HCl (0.01 mol/liter, pH 7.7) and sodium chloride (0.3 mol/liter) in the reservoir flask. Effluent flow rate, 40 ml/h. 6-ml fractions collected. Enzyme activity detected only in fractions emerging after 20 ml
zymic activity that resulted on Sephadex A-50 chromatography were pooled and concentrated; accordingly, it is not surprising that electrophoresis of Sephadex A-50 purified enzyme gave just one broad peak.

Figure 4 illustrates the crystals of the enzyme obtained from the Sephadex A-50 purified Genetron extract that was stored for about 4 weeks at 4°C. The crystals become yellow on addition of 1 drop of 1 mmol/liter p-nitrophenylphosphate in 0.1 mol/liter Tris buffer, pH 7.4, indicating a conversion of enzyme-substrate complex to the colored product, p-nitrophenol.

Figure 5 shows the immunodiffusion plate. The central well of this plate contained rabbit antiserum concentrated after treatment with Rivanol.

Discussion

Fewer steps are involved with the Genetron extraction and purification procedure as compared with the butanol extraction and purification procedure. Though the yield of protein was comparable in the preparation obtained by both procedures, the specific activity of the Genetron-extracted and purified enzyme was three times higher than that of the butanol-extracted and purified enzyme. The lower specific activity of the butanol-purified enzyme is due to the use of ammonium sulfate for precipitating the enzyme. This seems reasonable in view of the current concept that ammonium sulfate coordinates with zinc, which is present in the active center of enzyme molecule (8).

It is interesting that crystals appeared only in the "Genetron-purified" enzyme stored at 4°C for four weeks. No crystals appeared in the DEAE Sephadex-purified butanol-extracted enzyme regardless of the time it was stored at 4°C.

Genetron-113, the reagent first used as a "deproteinizing" agent in the purification of influenza virus (9), and used since then by many workers in the purification of viruses (10, 11), seems admirably suited to extracting the membrane-bound
phosphomonoesterase from human intestinal mucosa.

It is known that Genetron retains lipid and deproteinized material in the organic phase (9). Thus it apparently releases the enzyme in the same manner as does butanol—by disrupting the lipoprotein association and releasing the enzyme into the aqueous phase—and so the difference between the butanol and Genetron extraction procedures may lie in the complete removal of lipid material by the latter procedure.

Electrophoresis of the crude Genetron extract, and the slightly heterogenous profile obtained on DEAE Sephadex chromatography of the Genetron extract is consistent with the isoenzymic nature of phosphomonoesterase.

The fact that only the major peak was present in the DEAE Sephadex-purified Genetron extract might explain the single precipitation line obtained when the antiserum, freed from non-gamma-globulin proteins by treatment with Rivanol, was concentrated and used in the immuno-gel diffusion experiments.

From data presented in this paper, we conclude that phosphomonoesterase can be extracted from human intestinal mucosa and purified in a three-step procedure by use of trichlorotrifluoroethane (Genetron 113). The enzyme yield with this procedure (calculated from data presented in Table 2) is 34.2%, in contrast to the 5.7% enzyme yield with the butanol (2) procedure.

References