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PURIFICATION AND PROPERTIES OF HUMAN PLACENTAL ACID PHOSPHATASE III

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SUMMARY

Human placental acid phosphatase III has been purified 5000-fold from the crude tissue homogenate by a combination of gel filtration and ion-exchange chromatography. The resultant material had a specific activity of 10 international units *p*-nitrophenyl phosphate phosphatase activity per mg protein at 37°, and a pH optimum of 5.2–5.3 in sodium citrate. In sodium acetate buffer the pH curve was flattened and its optimum shifted to pH 4.8–5.0. Addition of 6-ethylmercaptopurine to the reaction mixture greatly stimulated the hydrolysis of *p*-nitrophenyl phosphate without further changing the pH curve. Purified enzyme III is moderately sensitive to gluteraldehyde and extremely sensitive to inactivation by heavy metal ions. These and other physico chemical properties of this enzyme are discussed in relation to difficulties in its histochemical visualization, possible uniqueness in human tissue, and similarities to an enzyme found in rabbit heterophil leukocytes.

INTRODUCTION

The human placenta from early gestation through term contains three chromatographically distinct acid phosphohydrolases having pH maxima between 4 and 5.8. These enzymes, which have been termed I, II and III, are largely solubilized by homogenizing placental villus tissue in isotonic sucrose, and are easily separable on the basis of their molecular size[1]. Enzymes I and II display broad although different substrate specificities consistent with the general classification of orthophosphoric monoester phosphohydrolases (EC 3.1.3.2), while enzyme III, an apparently non-lysosomal form, was found to hydrolyze relatively few of the common phosphate esters. Thus, it was felt that enzyme III perhaps should not properly be considered as one of the above class of enzymes[2]. Among other unique properties of this enzyme, we have previously reported a remarkable hydrolytic activity toward certain steroid phosphates such as estradiol-3-phosphate, as well as a several fold stimulation of *p*-nitrophenyl phosphate hydrolysis in the presence of N^6-substituted purines[2,3]. The

Abbreviation: TES, sodium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate.

present communication describes a partial (5000-fold) purification procedure for enzyme III from human term placentas as well as some kinetic studies with the purified preparation. An abbreviated report of this work has been presented elsewhere[1].

EXPERIMENTAL PROCEDURE AND RESULTS

Solubilization of enzyme

Fresh term placentas were perfused through the umbilical vein with 1 l of ice-cold 0.9% NaCl to wash out the fetal blood, followed by dissection of about 100 g of predominantly villus tissue from those areas blanched by the perfusion. The tissue was then briefly washed with cold 0.25 M aqueous sucrose and homogenized with 200 ml of this solution for 2 min in a Waring-type blender at high speed. The homogenate was centrifuged twice, once at 600 × *g* for 15 min to remove the bulk of insoluble material, and again at 105 000 × *g* for 40 min.

Chromatography on Sephadex G-75

The entire high speed supernatant was applied to a 5 cm × 85 cm column of Sephadex G-75 (40–120 μm) equilibrated with 0.01 M sodium *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonate, 0.001 M EDTA, pH 7.0 (TES–EDTA buffer). This column, operated at 4° at a flow rate of 1–2 ml/min, effectively separated phosphatases I and II from phosphatase III, the latter being eluted just behind a reddish-colored protein band which serves as a convenient marker. 25-ml fractions were collected and assayed at 37° as previously described using *p*-nitrophenyl phosphate[1], and those containing more than 10 munits/ml enzyme activity were pooled. Concentration of the pooled fractions was effected by adding 60 g of solid $(NH_4)_2SO_4$ (Mann, enzyme grade) to every 100 ml of protein solution, swirling to dissolve the salt, and allowing the protein to precipitate overnight in the cold. The precipitate, containing all of the enzyme, was centrifuged at 500 × *g* for 30 min and redissolved in 5–10 ml of TES–EDTA buffer for temporary storage at 4°. About 15–20 units of enzyme activity are commonly recovered at this stage. The entire procedure is then repeated 3 or 4 times to accumulate 50–75 units for the next step.

Chromatography on DEAE-Sephadex

This ion exchanger was obtained from the Sigma Chemical Co. (A-50, coarse) and washed with 0.5 M NaOH, water, and then 0.5 M HCl before adjusting to pH 7.0. The washed gel was equilibrated with 0.05 M Tris chloride, 0.001 M EDTA (pH 7.0 at 25°), and packed in a 2.5-cm column to a depth of 30 cm. The concentrated enzyme solution from the Sephadex G-75 column was submitted to buffer exchange with the above Tris–EDTA buffer on a column of Biogel P-6 (50–150 mesh) prior to adsorption on DEAE-Sephadex at 4°. After applying the enzyme, the DEAE-Sephadex column was washed with 150 ml of Tris–EDTA containing 0.2 M KCl, and the enzyme was then eluted by raising the KCl concentration to 0.6 M. The fractions containing enzyme were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, then submitted to buffer exchange on Biogel P-6 with TES–EDTA, pH 7.0. The use of other salts, such as $(NH_4)_2SO_4$ or NaCl, for elution of the enzyme from DEAE-Sephadex resulted in loss of activity and enzyme instability on standing. However, when eluted with KCl the

enzyme was found to be stable in cold solution for several weeks and could be precipitated with $(NH_4)_2SO_4$ for even greater stability. The enzyme is also stable in 0.05 M sodium citrate, pH 4.3, for at least several days.

Chromatography on CM-Sephadex

It is essential that this gel be precycled by stirring for at least 4 h with 0.5 M HCl, washed well, treated for an equal time with 0.5 NaOH, and then washed exhaustively with deionized water before adjustment of pH and buffer equilibration. Failure to adhere to this regimen has been found to result in irreversible loss of almost all of the enzyme activity on application to the gel. After washing, the CM-Sephadex was adjusted to pH 6.5 and equilibrated with TES–EDTA (pH 6.5 at 25°). The gel was then packed into a 2 cm × 20 cm column which was operated at 4° at a flow rate of about 0.5 ml/min. 30–50 units of enzyme activity in about 15 ml of TES–EDTA, pH 6.5, was applied to this column and eluted without delay with 25 ml of the same buffer containing 0.2 M KCl. A peak enzyme specific activity of about 10 units/mg protein was commonly obtained in the eluent. Protein was determined by

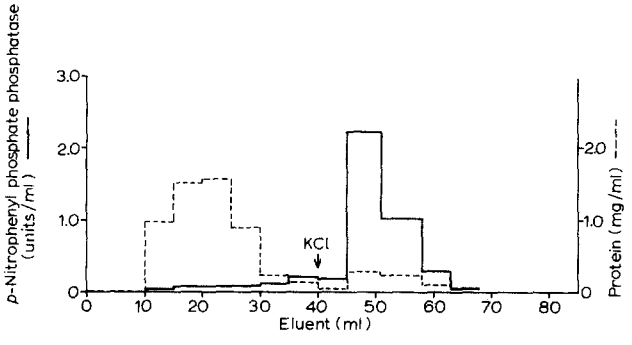

Fig. 1. Chromatography of placental phosphatase III on CM-Sephadex. In this experiment, 32 units of enzyme were placed on a 1.6 cm × 20 cm column and the eluent collected in approximately 5-ml fractions. KCl (0.2 M) was added to the buffer where indicated, and 26 units of activity were recovered with a peak specific activity of 8. See text for further details.

the method of LOWRY *et al.*[5], using crystalline bovine serum albumin as a standard. A typical elution pattern from CM-Sephadex is illustrated in Fig. 1. In this case 81% of the enzyme was recovered, and the bulk of this (70%) was eluted with the KCl. In some experiments we have found that a greater portion, sometimes as much as half, of the enzyme will fail to adhere to the gel and elute with the inactive protein peak. Occasionally in other experiments more than 90% of the enzyme has been lost in this step, presumably by irreversible adsorption to the ion exchanger. We feel that both of these problems can be circumvented by proper washing and recycling of the gel prior to use.

A summary of the individual purification steps and the resultant average specific enzyme activities is presented in Table I. An overall purification factor of 5000 was achieved starting from the low speed supernatant and assuming that the enzyme is completely solubilized; the procedure yields a 1000-fold purification from the high speed supernatant. The enzyme of specific activity 10 eluted from the CM column is unstable in the eluting buffer solution, losing activity over a period of a

TABLE I

PURIFICATION OF HUMAN PLACENTAL ACID PHOSPHATASE III

See text for details of the individual procedures.

Procedure	*Specific activity (units/mg protein at 37°)*	*Recovery (%)*
600 × *g* · 15 min supernatant	0.002–0.004	(100)
105 000 × *g* · 40 min supernatant	0.01	100
Sephadex G-75	0.1–0.7	90
DEAE-Sephadex	1.0	80–90
CM-Sephadex	7–10	50–80

few weeks when stored either at 4° or frozen. We have so far been unsuccessful in our attempts to stabilize it at this point by addition of SH compounds, $(NH_4)_2SO_4$, by precipitation or lyophilization.

Kinetic experiments with the partially purified enzyme

pH studies. Using a relatively crude preparation of enzyme III, we have previously reported[1] a pH optimum of 5.5 for *p*-nitrophenyl phosphate hydrolysis in sodium citrate buffer. With our present purified material we find a sharper pH optimum which is closer to 5.2 or 5.3, using saturating concentrations of this substrate in the same buffer, as illustrated by the curve in Fig. 2. When the reaction is carried out in 0.10 M sodium acetate buffer as in Fig. 3A, a substantially flattened-out curve is obtained with peak activity between pH 4.8 and 5.0. The mechanism of displacement of the pH curve by acetate ion is not clear in this case, although anion-induced displacements have been reported[6]. As mentioned previously, the hydrolysis rate of *p*-nitrophenyl phosphate by enzyme III is stimulated several fold by N^6-substituted purines. That this stimulation is not accompanied by a pH shift is demonstrated by a comparison of Curves A and B in Fig. 3.

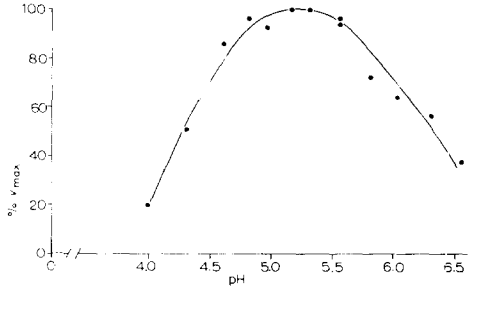

Fig. 2. pH–activity curve in citrate. The enzyme was incubated in 0.05 M sodium citrate buffer in the presence of saturating (0.009 M) concentrations of *p*-nitrophenyl phosphate.

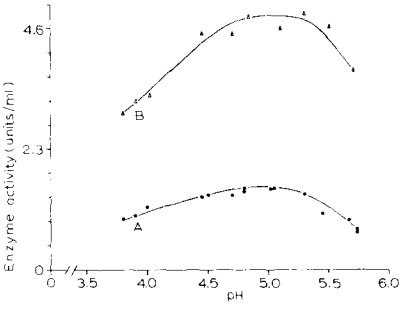

Fig. 3. pH–activity curve in acetate. 10-µl aliquots of enzyme solution were incubated for 10 min at 37° with 4.5 mM *p*-nitrophenyl phosphate in 0.1 M sodium acetate, in the presence (Curve B) and the absence (Curve A) of 1 mM 6-ethylmercaptopurine. Reaction velocities are expressed as units of activity per ml of enzyme solution.

Inhibitor studies. The following experiments were carried out in order to determine the feasibility of histochemical localization of enzyme III at the electron microscope level[7]. It was previously reported that this enzyme is extremely sensitive to the thiol-inactivating compound, *p*-chloromercuribenzoate, but unaffected by other phosphatase inhibitors such as L(+)-tartrate, molybdate or fluoride[1]. Heavy metal ion inhibition was not investigated, although the formation of insoluble heavy metal phosphates is a commonly used method for visualization of tissue phosphatase activity. In the present study we have found that the sensitivity of the soluble enzyme to heavy metal ions is largely a function of protein concentration: the preparations of highest specific enzyme activity being the most rapidly inactivated. Also, it was evident that some heavy metal ions are more potent inhibitors than others, other things such as protein, buffer and substrate concentrations being equal. For example, 1.0 mM Pb^{2+} completely inhibited the hydrolysis of *p*-nitrophenyl phosphate (1.0 mM) in 7.0 mM acetate buffer by an enzyme III preparation of specific activity 1.0. In this same system, as little as 0.05 mM Pb^{2+} inhibited the enzyme 50%. The fact that the Gomori histochemical procedure[8] for acid phosphatases calls for the use of 4 mM lead nitrate in the incubation medium is pertinent here. Ni^{2+} and Co^{2+} were only slightly less inhibitory: about 10% enzyme activity remaining in the presence of a 1.0 mM concentration of either ion. Under these same conditions even the Group IIA metals, magnesium, calcium and beryllium were inhibitory to an extent of 25, 50 and 90% respectively.

Since buffered gluteraldehyde is commonly used as a tissue fixative, we determined the enzyme's sensitivity both to this aldehyde and to dimethylarsinite buffer, as follows: an enzyme preparation of specific activity 1.0 was preincubated for 30 min at 0° with 0.15 M sodium dimethylarsinite, pH 7.3, and separately in a 0.1 M solution of this buffer containing 4% gluteraldehyde, followed by determination of residual enzyme activity. Although trivalent arsenicals are known to inactivate thiol groups by mercaptide formation[9], the pentavalent arsenic in the above buffer was without effect on the enzyme activity. The buffered gluteraldehyde, however, caused a 43% loss of activity, under the above conditions.

In view of the essential role of thiol groups for the activity of this enzyme one would expect the thiol-alkylating reagent, iodoacetate[10] to be inhibitory, which it is. Preincubation of enzyme for 10 min at 37° with 1.0 mM sodium iodoacetate buffered at pH 7.0 resulted in 30% loss of activity; in 10 mM inhibitor, over 95% of the enzyme was inactivated under these conditions. Addition of 1.0 mM 6-ethylmercaptopurine to the preincubation solution containing iodoacetate did not protect the enzyme from inactivation. This result was not surprising since the stimulation of enzymic hydrolysis of *p*-nitrophenyl phosphate by the purine is dependent on both the substrate and stimulator being present together in the assay medium. We suspect that *p*-nitrophenyl phosphate first forms a charge transfer complex with 6-ethylmercaptopurine, and this complex in turn reacts with the enzyme in an accelerated fashion. Studies are planned for the near future to elucidate this interesting point.

DISCUSSION

Previous investigations concerning the acid phosphohydrolase activity of human

placenta have been largely limited to histochemical observations[11–13] and a few biochemical studies on crude tissue extracts[14–16]. As regards the histochemical work, we feel that these studies require re-evaluation in the light of our recent demonstration of the heterogeneity of the placental enzyme activity, and especially the different substrate requirements of the individual enzymes[1–4]. For example, it is now questionable whether enzyme III has yet been visualized histochemically since it hydrolyzes the common phosphatase substrates poorly or not at all, and, as we have presently shown, is susceptible to rapid and complete inactivation by one-quarter of the standard concentration of lead ions used in the GOMORI[8] histochemical procedure. A similar situation has been reported in which one of the several acid phosphatase isoenzymes in maize was found to be specifically inhibited by a diazonium salt used for its histochemical localization[17].

Fractionation of a soluble extract of human placenta by starch gel electrophoresis has yielded four bands of α-naphthyl phosphate phosphatase activity[18]. Since enzyme III will not readily hydrolyze α-naphthyl phosphate[1] it is possible that there may be as many as five different placental acid phosphohydrolases. To further complicate the situation, this same group of workers has recently described a rare placental varient which yielded no fewer than seven distinct electrophoretic bands of α-naphthyl phosphate phosphatase activity[19]. In attempting to evaluate these results in relationship to our observations it may help to consider the acid phosphatase isoenzyme distribution found in human prostate. This tissue has yielded two forms by ion-exchange chromatography, four by immunological methods, and about twenty bands of activity using starch gel electrophoresis[25]. It is thought that the enzyme's charge heterogeneity is a result of a single enzyme protein with different numbers of sialic acid residues. Thus, the multiple forms of α-naphthyl phosphate phosphatase found in human placenta may simple be charge varients of enzymes I and II, both of which readily hydrolyze this substrate. One must also keep in mind the possibility of enzyme dissociation artifacts, either during the extraction procedure or as a result of the electric field. In this regard, fractionation by the relatively milder procedure of gel filtration may be a preferable method of obtaining unaltered enzyme for subsequent biochemical characterization.

To our knowledge, no acid phosphohydrolase of human origin has been purified to homogenity, although the human erythrocyte[20–22] and prostatic[23–25] enzymes have been intensively studied. Acid phosphohydrolyases from bovine liver and rat liver have, however, been obtained in an apparent homogeneous state: the former[26] was reported to have a specific enzyme activity of between 50 and 60, while the latter[27], which had also been crystallized, had a maximum specific activity of 4. An enzyme from *Escherichia coli*, also homogeneous, has been reported to have a specific activity of 17–18 (see ref. 28). (All of the specific enzyme activities quoted in this paper are expressed in international units and refer to the hydrolysis of *p*-nitrophenyl phosphate at 37°.) Our placental enzyme, although not homogeneous, would seem to be sufficiently purified for preliminary characterization studies such as are reported in this communication. Moreover, there was no evidence during ion-exchange or molecular sieve chromatography of further separation of activity into discrete fractions.

The question as to the physiologic substrate(s) for this enzyme remains unresolved, and although the hydrolysis of certain steroid phosphates is still a possibility,

we have been unable to demonstrate any 17β-estradiol:ATP transphosphorylase activity in human placenta, human fetal liver, or fetal adrenal tissue[4]. These results, of course, do not rule out the biosynthesis of steroid phosphates via other as yet unrecognized enzymic pathways, or under different experimental conditions. Nevertheless, the alternate possibility must also be considered: namely, that enzyme III is not unique to the human placenta; that is to say, that this is a species-specific rather than an organ-specific enzyme[29]. In this case we may expect to find an enzyme in some other human tissue having similar or identical properties. The acid phosphatases found in prostate, of which there are at least two[25], can be ruled out since all are strongly inhibited by L(+)-tartrate, while the placental enzyme is not. However, the three isoenzymes found in human erythrocytes have not yet been sufficiently characterized to rule out a possible identity, although one of these can perhaps be eliminated on the basis of its fluoride sensitivity[22]. Similarly, meaningful comparisons with other human acid phosphohydrolases must await their isolation and characterization.

Interestingly enough, the properties of placental enzyme III bear a striking resemblance to an acid phosphohydrolase found not in human tissue, but in rabbit heterophil leukocytes[30,31]. These articles describe a soluble, thiol-dependent acid *p*-nitrophenyl phosphatase which is unaffected by fluoride or L(+)-tartrate and is "clearly different from the lysosomal acid phosphatase". This enzyme has little or no hydrolytic activity toward β-glycerophosphate or glucose 6-phosphate. Thus far, the foregoing might be equally descriptive of placental enzyme III, although kinetic and physicochemical data for the leukocyte enzyme are as yet lacking. By way of contrast, a soluble acid phosphohydrolase with diametrically opposite properties has been partially purified from tobacco leaves[32]. This enzyme is resistant to inactivation by heavy metal ions up to 5 mM, as well as thiol inhibitors, but is quite sensitive to fluoride, L(+)-tartrate and ammonium molybdate. It will readily hydrolyze a wide variety of phosphomonoester bonds, including hexose phosphates and phenyl phosphate, and also some phosphate anhydride bonds. Thus, the physiologic function, natural substrate(s) and intracellular localization of these apparently non-lysosomal[33,34] acid phosphohydrolases remain intriguing questions, the answers to which may well modify our current concepts regarding this diverse class of enzymes.

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