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Oxidation of Polyamines by Diamine Oxidase from Human Seminal Plasma

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1. Diamine oxidase [amine–oxygen oxidoreductase (deaminating) (pyridoxal-containing), EC 1.4.3.6] was purified from human seminal plasma more than 1700-fold. The enzyme appeared to be homogeneous on polyacrylamide-gel electrophoresis at two different pH values. 2. The general properties of the enzyme were comparable with those described for other diamine oxidases from different mammalian sources. The molecular weight of the enzyme was calculated to be about 182000. 3. The enzyme had highest affinity for diamines, but polyamines spermidine and spermine were also degraded at concentrations that can be considered physiological in human semen. 3. The possible degradation of spermine by diamine oxidase in human semen *in vivo* may give rise to the formation of cytotoxic aldehydes that conceivably can influence the motility and survival of the spermatozoa.

One of the unique properties of human semen is its high content of the polyamine spermine as compared with other body fluids and tissues (Weaver & Herbst, 1958; Mann, 1964; Fair *et al.*, 1972; Jänne *et al.*, 1973). The physiological function, if any, of spermine in the semen of most mammalian species is not known. Spermine does not appear to have either beneficial or deleterious effect on the motility or metabolism of the mammalian spermatozoa at least when added *in vitro* (Mann, 1964; Williams-Ashman & Lockwood, 1970).

In 1941 Zeller (Zeller, 1941; Zeller & Joël, 1941) reported that human semen contains high diamine oxidase activity, and besides diamines endogenous spermine also appeared to be slowly oxidized in human seminal plasma. The fact that spermine can be oxidized in human semen is not only interesting from the enzymological point of view but might be of physiological importance, as the formation of cytotoxic oxidized derivatives of polyamines (Tabor & Rosenthal, 1956; for further references see also Bachrach, 1970*a,b*) *in vivo* could profoundly influence the metabolism and fertilizing capacity of the postejaculatory human spermatozoa.

In a recent survey we confirmed Zeller's (1941) primary observation that human semen is indeed a rich source of diamine oxidase (Jänne *et al.*, 1973), and we also found some indirect evidence that spermine and spermidine could possibly be oxidized by the enzyme.

In the present paper we have further purified and characterized diamine oxidase from human seminal plasma. In many respects the general properties of the enzyme are similar to those described for other diamine oxidases from various mammalian sources. The enzyme also appears to degrade spermine and spermidine, with the formation of products that resemble the iminoaldehydes that arise from the oxidation of polyamines by the amine oxidase (spermine oxidase) from ruminant serum.

Experimental

Collection of seminal plasma

The semen samples were obtained from a local clinical laboratory performing semen analyses. Spermatozoa were separated by low-speed centrifugation and the seminal plasma was stored at −20°C until used.

Chemicals

[1,4-^{14}C]Putrescine (sp. radioactivity 17.5mCi/mmol), [1,4-^{14}C]spermidine (sp. radioactivity 9.82mCi/mmol) and [1,4-^{14}C]spermine (sp. radioactivity 18.75mCi/mmol) were purchased from the New England Nuclear Corp., Dreieichenhain, Germany. Before use radioactive spermidine and putrescine were purified on a Dowex 50 (H^+ form) column. On paper electrophoresis (Raina, 1963) labelled spermine appeared to be free of any radioactive contaminant. Unlabelled putrescine, spermidine and spermine were purchased from Calbiochem, San Diego, Calif., U.S.A. Unlabelled spermine was found to contain about 0.9mol of material/100mol that migrated on paper electrophoresis like spermidine, and 0.1–0.2mol of material/100mol that appeared to be putrescine. This minor contamination of spermine, however, was not of any practical

significance. Histamine was purchased from E. Merck, Darmstadt, Germany and cadaverine from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Analytical methods

Putrescine, spermidine and spermine were measured after butanol extraction on paper electrophoresis (Raina, 1963; Raina & Cohen, 1966). Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Polyacrylamide-gel electrophoresis was carried out at 4°C essentially as described by Davis (1964). The following method described by Hampton *et al.* (1972) was used to locate the amine oxidase activity (the formation of H_2O_2) on the polacrylamide gels. After electrophoresis the gels were incubated overnight in the dark at 20°C in small test tubes containing 25mM-Tris–HCl buffer, pH7.4, and 0.02% *o*-dianisidine (Fluka AG, Buchs, Switzerland), 0.1% (w/v) horseradish peroxidase (crude; Sigma Chemical Co., St. Louis, Mo., U.S.A.) in the presence of different amines as the substrates.

The sucrose-density-gradient centrifugations were carried out in a Spinco L-3 ultracentrifuge with a SW 50 rotor. Linear 5–20% (w/v) sucrose gradients in 25mM-Tris–HCl buffer, pH7.4, were made with a gradient former from Beckman Instruments. Bovine liver catalase (crude, Sigma Chemical Co.) and yeast alcohol dehydrogenase (Boehringer und Soehne, G.m.b.H., Mannheim, Germany) were used as markers in the centrifugations.

The molecular Stokes radius of diamine oxidase was calculated as described by Ackers & Steere (1967). The Sephadex G-200 column was calibrated with the aid of Blue Dextran 2000 (Pharmacia, Uppsala, Sweden), potassium dichromate, bovine liver catalase (see above), yeast alcohol dehydrogenase (see above) and bovine serum albumin.

The double-reciprocal lines were computed by the least-squares method.

Assay of diamine oxidase activity

Diamine oxidase activity was assayed by the method of Okuyama & Kobayashi (1961) as modified by Tryding & Willert (1968) with [^{14}C]putrescine as the substrate, and by extracting the radioactive pyrroline formed with a toluene-based scintillator (Tryding & Willert, 1968). The extraction recovery of two subsequent extractions was found to be 70% of all extractable radioactive material and appropriate corrections were made to the results. The radioactivity was converted into nmol of pyrroline formed by using [^{14}C]putrescine as the internal standard.

Diamine oxidase activity was also assayed spectrophotometrically by measuring the formation of H_2O_2 in the presence of different amines as substrates essentially as described earlier (Aarsen & Kemp, 1964; Gunther & Glick, 1967). The amount of H_2O_2 formed during the incubation was calculated from a standard curve made under duplicating conditions in the presence of various amounts of H_2O_2 (obtained from E. Merck AG, and titrated with $KMnO_4$ before use). By using putrescine as the substrate a good stoicheiometry between the formation of H_2O_2 and toluene-extractable material from [^{14}C]putrescine was obtained.

Purification of the diamine oxidase activity from human seminal plasma

Seminal plasma (420ml) separated from the spermatozoa was fractionated with solid $(NH_4)_2SO_4$ (specific enzyme grade, Schwarz/Mann, Orangeburg, N.Y., U.S.A.) at 0°C. The proteins precipitated between 0.45 and 0.70 (w/v) saturation of $(NH_4)_2SO_4$ were dissolved in a small volume of 25mM-Tris–HCl buffer, pH7.4, containing 0.1mM-EDTA (standard buffer) and dialysed overnight against the same buffer. The dialysed $(NH_4)_2SO_4$ fraction (fraction 2) was subjected to chromatography on a column (3cm×40cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with the standard buffer. After the application of the sample the column was washed with 100ml of the equilibration buffer and connected to a linear gradient of 0.1–0.25M-NaCl (gradient vol. 1000ml). Diamine oxidase activity was eluted from the column at about 0.2M-NaCl. The most active fractions were pooled (fraction 3; 86ml) and applied to a hydroxyapatite column (3cm×12cm; Hypatite C, Clarkson Chemical Co. Inc., Williamsport, Pa., U.S.A.) equilibrated with the standard buffer without EDTA. The column was eluted with a potassium phosphate gradient (in the standard buffer without EDTA) from 0.1 to 0.4M (gradient vol. 500ml). Diamine oxidase activity was eluted from the column at about 0.15–0.12M-potassium phosphate. The most active fractions were pooled (fraction 4; 44ml) and concentrated in an Amicon ultrafiltration cell to 7.0ml. The concentrated hydroxyapatite fraction was applied to a column (2.6cm×60cm) of Sephadex G-200 previously equilibrated with the standard buffer containing, in addition, 100mM-KCl. Diamine oxidase activity was eluted from the column at about 1.34 times the void volume. The three most active fractions were pooled (12.0ml; fraction 5) and concentrated again in an ultrafiltration cell. A further purification of diamine oxidase was achieved by polyacrylamide-gel electrophoresis. The concentrated fraction 5 was divided into several portions (0.3ml) and subjected to polyacrylamide-gel electrophoresis essentially as described by Davis (1964). The electrophoresis was carried out at 4°C with a constant current

of 2.5mA/tube for about 2h. One of the tubes was stained with Coomassie Brilliant Blue and one tube was incubated in the presence of *o*-dianisidine and peroxidase (see under 'Analytical methods') to locate the diamine oxidase activity on the gel. With the aid of the Coomassie Brilliant Blue and *o*-dianisidine stainings it was possible to cut the rest of the gels so that only diamine oxidase was in the gel piece. The pieces were minced and eluted overnight with the standard buffer containing 100mM-KCl. After centrifugation for 15min at 10000*g* the supernatant fraction was separated and designated fraction 6.

Results

General properties of the seminal plasma diamine oxidase

The purification steps of the diamine oxidase activity from human seminal plasma are demonstrated in Table 1. The six-step procedure resulted in a final purification of more than 1700-fold as compared with the crude seminal plasma with an overall yield of 1.2%. Fraction 6 preparation appeared to be homogeneous on polyacrylamide-gel electrophoresis at pH8.6 and 7.4. At pH8.6 the single protein band on the gels (Coomassie Brilliant Blue-stained) was closely associated with the band showing the formation of H_2O_2 when the gels were incubated in the presence of either putrescine or spermine, and *o*-dianisidine and peroxidase.

The pH optimum of the enzyme was very close to 7.0. The sedimentation coefficient of diamine oxidase was calculated to be 8.6S by using catalase (11.2S) and yeast alcohol dehydrogenase (7.6S) as markers in the sucrose-density-gradient centrifugations. From the Sephadex G-200 molecular sieving data it was calculated that the molecular Stokes radius of the enzyme was 4.81nm. By assuming a partial specific volume of 0.74 an apparent molecular weight of 182000 was obtained for the active form of diamine oxidase from human seminal plasma.

The enzyme activity was inhibited by carbonyl reagents such as isonicotinic acid hydrazine, semicarbazide and canaline. In no case, however, could the enzyme activity be restored by the addition of exogenous pyridoxal 5′-phosphate.

Oxidation of various amines by the diamine oxidase

In Table 2 the relative formation of H_2O_2 in the presence of different amines as substrates is shown during the purification of the enzyme. As shown, the

Table 1. *Purification of diamine oxidase from human seminal plasma*

Seminal plasma (420ml) was separated from the spermatozoa and processed as described in the text. The activities are expressed as nmol of product formed from putrescine in 30min.

Fraction	10^{-3}×Total activity (nmol)	Total protein (mg)	Specific activity (nmol/mg of protein)	Purification (fold)
1. Seminal plasma	80.4	33000	2.4	1
2. $(NH_4)_2SO_4$ fraction	33.0	8300	4.0	1.7
3. DEAE-cellulose	29.7	640	46	19
4. Hydroxyapatite	15.4	60	257	107
5. Sephadex G-200	5.6	2.0	2770	1150
6. Gel electrophoresis	1.0	0.23	4280	1780

Table 2. *Relative formation of H_2O_2 in the presence of various amines during the purification of the diamine oxidase from human seminal plasma.*

The enzyme preparations from human seminal plasma were incubated in the presence of 1mM-putrescine, -spermidine, -spermine or -histamine. The results are expressed as the production of H_2O_2 relative to that achieved with putrescine as the substrate. H_2O_2 formation was measured as described in the text.

	Production of H_2O_2 in the presence of			
Enzyme preparation	Putrescine	Spermidine	Spermine	Histamine
1. Seminal plasma	1.00	0.94	0.64	0.51
2. $(NH_4)_2SO_4$ fraction	1.00	1.14	0.63	0.64
3. DEAE-cellulose	1.00	0.91	0.46	0.38
4. Hydroxyapatite	1.00	0.57	0.46	0.29
5. Sephadex G-200	1.00	0.46	0.46	0.27
6. Gel electrophoresis	1.00	0.53	0.42	0.32

ratio of the oxidation of spermine to that of putrescine remained relatively constant during the whole purification procedure, i.e. being 0.64 when the dialysed plasma and 0.42 when the fraction 6 preparation was used as the source of the enzyme. The ratio of the formation of H_2O_2 in the presence of spermidine to that in the presence of putrescine, however, decreased from about 1.0 in the seminal plasma and the $(NH_4)_2SO_4$ fraction to 0.53 in the final fraction 6 preparation. Similarly the ratio of the oxidation of histamine to that of putrescine also decreased during the purification. This might indicate that there are in human seminal plasma other enzymes capable of oxidative degradation of spermidine and histamine. The best substrate so far tested for the enzyme appeared to be cadaverine, and it was also found that diamine oxidase from human seminal plasma could not oxidize monoamines such as noradrenaline.

The enzyme showed highest affinity for putrescine with an apparent K_m value of 0.013 mM (Fig. 1). Fig. 1 also shows that spermine acted as a competitive inhibitor for putrescine with an apparent K_i of 0.13 mM.

Spermine and spermidine were also oxidized by the enzyme although the affinities were clearly lower than that for putrescine. The apparent K_m value for spermine was 0.1 mM, which is, as expected, reasonably close to the K_i value calculated for spermine as the competitive inhibitor for putrescine. The apparent K_m value for spermidine was about 5 times higher than that for spermine, i.e. 0.56 mM. By using either spermine or spermidine as the substrate it appeared that the maximum velocities obtained were about the same ($V_{max.}$ 870 nmol of H_2O_2/60 min per mg of protein) and approx. 50 % of that in the presence of putrescine or cadaverine as the substrate. The Lineweaver–Burk plots for both spermine and spermidine were linear. The production of H_2O_2 was linear for at least 60 min.

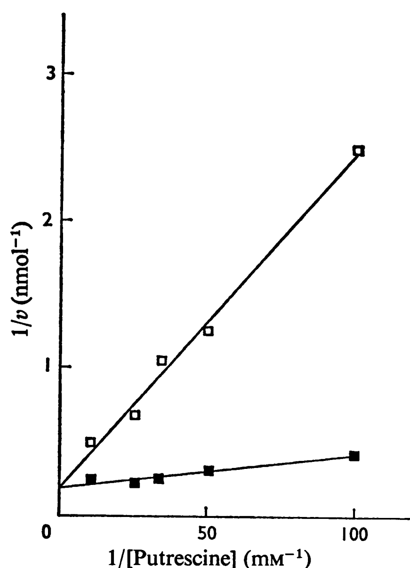

Fig. 1. *Lineweaver–Burk plot of the effect of putrescine on the diamine oxidase activity in the absence or presence of 1 mM-spermine*

The enzyme activity was determined with fraction 5 enzyme preparation (0.003 mg of protein) by using [^{14}C]putrescine as the substrate, and measuring the toluene-extractable radioactivity. The double-reciprocal lines were computed by the least-squares method. ■, No spermine; □, 1 mM-spermine.

Nature of the reaction products by seminal plasma diamine oxidase

When putrescine is used as the substrate for diamine oxidase an aminobutyraldehyde, with a concomitant release of H_2O_2, is formed. The aldehyde, however, is rapidly cyclized to Δ-pyrroline which is the final oxidation product (for references see Kapeller-Adler, 1970). The reaction product of a complete oxidation of spermine by the amine oxidase present in the blood plasma of ruminants appears to be a rather labile iminodialdehyde (Tabor *et al.*, 1954, 1964), which may rapidly undergo a series of secondary transformations such as β-elimination, polymerization (Tabor *et al.*, 1964; Kimes & Morris, 1971). To investigate the nature of the reaction products arising from the oxidation of spermine by human seminal plasma amine oxidase, a large-scale incubation was carried out with [1,4-^{14}C]-spermine as the substrate and a partially purified diamine oxidase preparation (fraction 3) as the enzyme. The incubation (60 min) was stopped with trichloroacetic acid and the acid-soluble supernatant fraction was applied to a Dowex 50 (H^+ form) column. The column was first washed with water and thereafter with 1 M-HCl (no radioactivity was eluted from the column with these washings) followed by an elution with 2 M-HCl. The radioactive fractions eluted with the 2 M-HCl were evaporated to dryness at 30–35°C under reduced pressure, dissolved in a small volume of water and a sample was subjected to paper electrophoresis as described by Raina (1963). As shown in Fig. 2, the major part of the radioactivity (solid line) appeared in two fractions, one migrating slightly more slowly than spermine and the other migrating with spermine, which was used as a marker during the electrophoresis (no spermine can be eluted from a Dowex 50 column by 2 M-HCl). Portions of the same residues were neutralized and kept for 15 min in a boiling-water bath, and after that they were subjected to paper electrophoresis under identical conditions. Fig. 2 shows that the boiling resulted in a

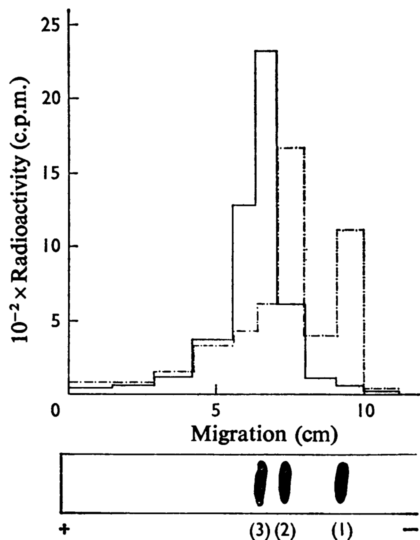

Fig. 2. *Paper electrophoretogram of the radioactive reaction products formed from [^{14}C]spermine by seminal plasma diamine oxidase*

[1,4-^{14}C]Spermine was incubated with partially purified diamine oxidase for 60min. After stopping the reaction with trichloroacetic acid, the reaction products were applied to a Dowex 50 (H^+ form) column. Radioactive products were eluted with 2M-HCl, a portion was kept in a boiling-water bath for 15min (see the text for details) and then both samples were subjected to paper electrophoresis. Putrescine (1) (fastest-migrating), spermidine (2) and spermine (3) were used as markers in the electrophoresis. ——, Radioactivity pattern without boiling; ·—·—·—·, radioactivity pattern after boiling.

change of the radioactivity pattern. Part of the radioactivity (dotted line) now corresponded to the migration of spermidine and another part migrated in the same place as putrescine. This finding agrees well with the observation by Tabor *et al.* (1964) indicating that the iminoaldehydes arising from the incomplete and complete oxidation of spermine are swiftly degraded on heating by a β-elimination reaction with the formation of spermidine and putrescine respectively. Thus the oxidation of spermine by diamine oxidase from human seminal plasma seems to result in the formation of reaction products similar to those arising from the oxidation of spermine by the amine oxidase (spermine oxidase) from the blood plasma of ruminants.

Discussion

Zeller (1941) was the first to describe the presence of high concentrations of diamine oxidase in human seminal plasma. He also stated that this enzyme activity appeared to oxidize spermine present in human semen. This was based on the observation that in the absence of cadaverine (used as the substrate for the reaction) undialysed semen (containing high concentration of spermine) catalysed a slow destaining of indigo disulphonate used as the chromogen. In a survey on polyamines and polyamine-metabolizing enzymes in human semen (Jänne *et al.*, 1973) we confirmed the high diamine oxidase activity in human semen. It also appears from the present results that human semen is able to degrade spermine with a concomitant formation of H_2O_2. The possibility that spermine normally present in human semen might be degraded to aldehyde products that are known to be extremely toxic for a variety of eukaryotic cells (Bachrach, 1970*b*), including human spermatozoa (Tabor & Rosenthal, 1956), might be important for the metabolism, survival and fertilizing capacity of the sperm cells. A high diamine oxidase activity in the semen together with a sufficient amount of spermine might have a deleterious effect on the motility of spermatozoa, assuming that this combination results in a formation of the cytotoxic aldehydes. In fact, we found a trend that supports this hypothesis. Table 3 shows the activity of diamine oxidase and the concentration of spermine in more than 100 semen samples. The diamine oxidase activity tended to be highest in semen samples showing the poorest initial motility, i.e. in those where less than 50% of the sperm cells were motile. The concentration of spermine was also lower in samples showing poor motility. Owing to the large variations and relatively small number of samples with low motility these differences, however, were not statistically significant. In any case, the actual concentration of spermine in human semen (about 3mM; Jänne *et al.*, 1973) and the kinetic parameters of diamine oxidase (K_m for spermine about 0.1mM) will certainly make the oxidation of spermine possible in human semen.

Table 3. *Relation of diamine oxidase activity and the concentration of spermine to the motility of spermatozoa in human semen samples*

The diamine oxidase activity was assayed by using [^{14}C]putrescine as substrate as described in the text. Results are means ± S.E.M. with the numbers of observations in parentheses.

Percentage of spermatozoa motile at initial count	Diamine oxidase activity (nmol/30min) per ml)	Spermine concentration (μmol/ml)
≤50	302 ± 130 (12)	2.33 ± 0.42 (12)
51–70	201 ± 35 (67)	3.02 ± 0.18 (67)
>70	165 ± 23 (24)	3.12 ± 0.32 (24)

From the enzymological point of view the general properties of diamine oxidase from human seminal

plasma resembled those described for other amine oxidases from various mammalian sources (for references see Kapeller-Adler, 1970). Bardsley *et al.* (1974) and Crabbe & Bardsley (1974) reported an extensive purification and characterization of diamine oxidase from human placenta. In many respects diamine oxidase from human seminal plasma corresponds to that from the placenta. In both enzymes the substrate spectrum was much alike except that Bardsley *et al.* (1974) reported that spermidine was not oxidized by their purified enzyme preparations but as shown in the present study spermidine can serve as a substrate for seminal plasma diamine oxidase.

The physiological significance of the diamine oxidase and/or spermine in human seminal plasma is not known. However, on the basis of the toxicity of oxidized polyamines for mammalian cells and especially the deleterious effect possessed by spermine together with the soluble amine oxidase from bovine blood on spermatozoa, a high diamine oxidase activity in human seminal plasma might conceivably influence the metabolism and survival of the post-ejaculatory spermatozoa.

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