Partial characterization of lysyl oxidase from several human tissues

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Lysyl oxidase activity was assayed in urea extracts of a number of human tissues, proving to be highest in skin. Antibodies to human placental lysyl oxidase completely inhibited the activity of crude lysyl oxidase from all the human tissues studied, with no significant differences in the amounts of antiserum required for 50% inhibition. By contrast, marked differences were found in this value between skin tissue samples from different species. The M_r of lysyl oxidase in crude extracts of human skin and in the medium of cultured human skin fibroblasts was 30000 by gel filtration, no active species with a higher M_r being detectable. Four forms of lysyl oxidase activity were seen in DEAE-cellulose chromatography of urea extract from human skin, all having M_r 30000. Antibodies to human placental lysyl oxidase stained a 30000- M_r protein in urea extracts of all the human tissues studied and in the medium of cultured human skin fibroblasts when examined by immunoblotting after sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis, but they also stained high- M_r material. The findings suggest that there are no immunologically distinct lysyl oxidase isoenzymes in the various human tissues and that the true M_r of lysyl oxidase in crude urea extracts is 30000.

Lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) is a copper enzyme that initiates the cross-linking of collagens and elastin by converting the amine side chains of specific lysine and hydroxy-lysine residues into aldehydes (see Siegel, 1979). The enzyme has been purified to near-homogeneity or homogeneity from several animal sources (see Siegel, 1979; Sullivan & Kagan, 1982) and human placental tissue (Kuivaniemi *et al.*, 1984), and two specific antisera have been prepared to the human placental enzyme (Kuivaniemi *et al.*, 1984). The M_r of lysyl oxidase has been reported to be about 30000 or 60000, but values of 80000 and 160000 have also been obtained, different M_r values having even been recorded for enzyme from the same sources (see Siegel, 1979). Preparations of lysyl oxidase from all the sources studied are heterogeneous, with multiple peaks of activity in DEAE-cellulose chromatography. The reasons for this are unknown, but investigations have indicated that there are no definite structural, catalytic or immunological differences between the various forms (Sullivan & Kagan, 1982; Kuivaniemi *et al.*, 1984).

One possible explanation for the conflicting data concerning the molecular properties of lysyl oxidase is that tissues may contain several isoenzymes

and that the purification procedures used may have selected one or other of these. The present work set out, therefore, to study the enzyme in crude tissue extracts. Special emphasis was laid upon the following questions. Are there distinct differences between the lysyl oxidase found in different human tissues or in different species when determined by inhibition studies with antibodies to human placental lysyl oxidase? What is the M_r of lysyl oxidase in crude extracts from various human tissues? Is lysyl oxidase heterogeneous when studied by DEAE-cellulose chromatography of crude human skin extract?

Experimental

Materials

The substrate of [$6\text{-}^3\text{H}$]lysine-labelled purified chick-embryo calvaria collagen and a collagen-agarose affinity column were prepared as described previously (Kuivaniemi *et al.*, 1984). Two specific antisera to human placental lysyl oxidase were available, one to total enzyme protein (antiserum T) and the other to lysyl oxidase purified from the DEAE-cellulose chromatography peaks 1 and 2 (antiserum I) (Kuivaniemi *et al.*, 1984). Both were immunoadsorbed on a

column containing a purified lysyl oxidase contaminant linked to agarose (Kuivaniemi *et al.*, 1984). The sources of the other materials were as described elsewhere (Peltonen *et al.*, 1983; Kuivaniemi *et al.*, 1984).

The human tissue pieces were obtained from those removed in various surgical operations, all the tissues being thus removed from the patients on medical indications. The placentas were obtained from normal deliveries and caesarian sections.

Preparation of tissue extracts and cell-culture media

The human tissue pieces were freshly minced with scissors and homogenized in 0.15M-NaCl/0.1M-sodium phosphate buffer, pH 7.8 (10ml/g of tissue) in an Ultra-Turrax homogenizer four times for 5s at 0°C. The homogenate was centrifuged at 15000g for 30min at 4°C. The supernatant, which did not contain any lysyl oxidase activity, was discarded, and the pellet was suspended in 6M-urea in 10mM-sodium phosphate buffer, pH 7.8 (2ml/g of tissue), stirred overnight at 4°C and centrifuged as above. The supernatant was used for the lysyl oxidase assay.

The human placental enzyme was prepared as described elsewhere (Kuivaniemi *et al.*, 1984). Because of the very low activity of lysyl oxidase in the urea extract of placenta, the placental enzyme used in activity assays with antiserum was purified by collagen-agarose chromatography and that used in immunoblotting by collagen-agarose and DEAE-cellulose chromatographies, all of these being performed as described previously (Kuivaniemi *et al.*, 1984).

Skin from a 6-month-old rabbit, a 5-month-old guinea pig, a 60-day-old rat, a 90-day-old mouse and 16-day chick embryos was homogenized as above, except that 10ml of 6M-urea/phosphate buffer was used per g of tissue.

Fibroblast cultures were established from human skin specimens, mouse 3T3 skin fibroblasts and smooth-muscle cells of bovine aorta. The cells were grown as described elsewhere (Peltonen *et al.*, 1983), and samples of the culture media were used to assay the lysyl oxidase activity (Peltonen *et al.*, 1983).

DEAE-cellulose and Sephacryl S-200 chromatographies of human lysyl oxidase

A sample of the urea extract from human skin was applied to a 60-ml DEAE-cellulose column equilibrated with 6M-urea in 10mM-sodium phosphate buffer, pH 7.8, at 4°C. The column was washed with 60ml of the equilibration buffer and eluted with an 80ml linear gradient of 0–0.5M-NaCl in the 6M-urea/phosphate buffer. Sephacryl S-200 chromatography was performed by applying a sample of the urea extract of human skin or

(NH₄)₂SO₄-precipitated culture medium of human skin fibroblasts to a column (1.5cm × 90cm) equilibrated and eluted with 6M-urea buffered as above.

Other methods

Lysyl oxidase activity was assayed as described previously (Peltonen *et al.*, 1983; Kuivaniemi *et al.*, 1984).

Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis was performed with 12%-acrylamide gels (King & Laemmli, 1971), and immunoblotting experiments after electrophoresis were performed as described previously (Kuivaniemi *et al.*, 1984). The lysyl oxidase activity in human tissues was expressed per mg of tissue protein assayed by the α -amino N method of Rubinstein & Pryce (1959). Tissue pieces (about 50mg) were hydrolysed in 2ml of 6M-HCl at 120°C for 20h, and the α -amino N content was determined in the hydrolysates. The protein content of the fractions in the various chromatographies was measured by determining A_{280} or A_{230} , with bovine serum albumin as the standard.

Results and discussion

Comparison of the amounts of lysyl oxidase activity in the human tissues

Human venous, cartilage, fascia, skin, pleura, lung and placental tissues were chosen for study here because they altogether contain collagen types I–VII (Bornstein & Sage, 1980; Bentz *et al.*, 1983; Hessle & Engvall, 1984) and because they are easily available in fresh form from surgical operations and deliveries. The enzyme preparations used were urea extracts of crude tissue homogenates. Skin had the highest lysyl oxidase activity when expressed per mg of protein. The values for the other tissues were, as percentages of that in skin: vena 83%, fascia 73%, pleura 47%, cartilage 38%, lung 37% and placenta 7%.

Immunological cross-reactivity between lysyl oxidases from different human tissues and between human and animal lysyl oxidases

The immunological studies were performed with antisera to the human placental enzyme, the only lysyl oxidase that had been purified from any human tissue (Kuivaniemi *et al.*, 1984). Two specific antisera were available, antiserum T (prepared to total lysyl oxidase protein) being more effective in inhibiting the native enzyme, and antiserum I (prepared to the enzyme purified from DEAE-cellulose chromatography peaks 1 and 2) being more effective in staining the denatured enzyme protein (Kuivaniemi *et al.*, 1984).

The human venous, cartilage, fascia, skin and

placental lysyl oxidases were compared with respect to their inhibition by antiserum T. This antiserum completely inhibited the activity of crude lysyl oxidase from all these tissues. The inhibition curves were mutually similar (as shown for the skin, venous and cartilage enzymes in Fig. 1a). Similarly, no significant differences were found between the tissues in the amounts of antiserum required for 50% inhibition of 1000 d.p.m. of lysyl oxidase activity (Table 1).

In order to determine whether the antibody inhibition approach used above could reveal any differences in cases of lysyl oxidases that are likely to be only slightly different, this same technique was used to study the immunological characteristics of lysyl oxidases from several animal sources. A considerable degree of species specificity was found, as the inhibition curves obtained were distinctly different from those of the human enzymes (as shown for the mouse and rat skin enzymes compared with the human skin enzyme in Fig. 1b). The highest degree of inhibition obtained was in most cases only about 40–50%, and the amount of antiserum required for 40% inhibition of 1000 d.p.m. of enzyme activity was 4–8-fold

higher with animal than with human enzymes (Table 2).

Human and animal lysyl oxidases have previously been reported to be very similar with respect to a number of molecular and catalytic properties (Stassen, 1976; Siegel, 1979; Sullivan & Kagan,

Table 1. *Inhibition of lysyl oxidase activity from certain human tissues by a specific antiserum*

Lysyl oxidase was assayed in tissue extracts or culture medium with various concentrations of antiserum T prepared to human placental lysyl oxidase. The value for the amount of antiserum required for 50% inhibition of 1000 d.p.m. of enzyme activity was obtained from the inhibition curves.

Source of enzyme	Antiserum required for 50% inhibition (μl)
Skin	6.0
Vena	7.0
Fascia	6.0
Cartilage (toe)	6.5
Placenta	5.8
Cultured-skin-fibroblast medium	6.0

Fig. 1. *Inhibition of the activity of crude lysyl oxidase (a) from human cartilage (●), skin (■) and venous (▲) tissue, and (b) from rat skin (●), mouse skin (■) and mouse-skin-3T3-cell medium (▲) by antiserum T*
Conditions were as described in the Experimental section. No inhibition was obtained with 0–10 μl of non-immune serum, whereas 20–50 μl gave an inhibition of about 5–15% for the human enzymes and an inhibition of about 10–20% for the animal enzymes. The inhibition of the activity of crude human skin lysyl oxidase observed in the same experiments is shown for comparison (○) in (b).

1982; Kuivaniemi *et al.*, 1984). The inhibition assay used in the present study thus seems to be able to detect differences between enzymes that are only slightly different. The failure to find any such differences between the lysyl oxidases in crude extracts from various human tissues therefore suggests that lysyl oxidase may not have any tissue-specific isoenzymes.

The absence of immunologically distinct lysyl oxidase isoenzymes suggests that antisera prepared against placental lysyl oxidase can also be used to study the enzyme in other human tissues. It would thus seem possible to use these antibodies to develop a radioimmunoassay for the enzyme protein. Such a method might also provide an opportunity to assay lysyl oxidase from serum samples, which has so far been difficult owing to the presence of inhibitors of the enzyme activity (Shieh *et al.*, 1977).

Molecular properties of crude lysyl oxidase from several human tissues

Sephacryl S-200 gel filtration of the crude skin

and skin-fibroblast-medium lysyl oxidases, carried out in 6M-urea, gave only a single peak of activity, the elution position of which corresponded to an M_r of about 30000 (result not shown). Immunoblotting performed with antiserum I after sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis likewise demonstrated the presence of a 30000- M_r protein in the urea extracts from all the tissues studied (as shown for cartilage, fascia and skin in Fig. 2) and in the medium of cultured skin fibroblasts (Fig. 2, lanes *e* and *f*). Immunoreactive material with high M_r values was also detected in all the tissue samples, however. The amount of the high- M_r material varied markedly in various experiments with the same tissues (Fig. 2, lanes *a* and *b*). If the samples were reduced before electrophoresis, the 30000- M_r band became more prominent, but the high- M_r material was still seen (results not shown). The high- M_r material thus probably consists at least in part of lysyl oxidase aggregates. This suggestion is supported by the findings that the high- M_r material blocked the antiserum from staining the lysyl oxidase protein

Fig. 2. Analysis of crude urea extract of human cartilage (lanes *a* and *b*), fascia (lane *c*) and skin (lane *d*), and culture medium from human skin fibroblasts (lanes *e* and *f*), by immunoblotting after sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis.

Conditions were as described in the Experimental section. The samples were not reduced, only denatured by heating. The arrows on the left indicate the positions of the protein standards used and seen after heparin/Toluidine Blue staining. The arrow on the right indicates the position of human placental lysyl oxidase. The samples were studied in three separate experiments, and the standards shown are in exactly correct positions only in the case of lanes *a* and *b*. The other slips have been placed so that the band corresponding to the lysyl oxidase standard is in all slips in the same position.

Table 2. Inhibition of lysyl oxidase activity from different species by antiserum T against the human placental enzyme

Lysyl oxidase was assayed in skin extracts or culture medium with various concentrations of antiserum T to human placental lysyl oxidase. Since not all the animal sources gave an enzyme activity that could be inhibited to 50%, the 40%-inhibition values were chosen for comparison. The amount of antiserum required for 40% inhibition of 1000d.p.m. of enzyme activity was obtained from the inhibition curves. The values shown are corrected for an inhibition of 5–20% obtained with 20–50 μ l of non-immune serum.

Source of enzyme	Antiserum required for 40% inhibition (μ l)
Human skin	3.5
Rabbit skin	16
Guinea-pig skin	20
Rat skin	28
Mouse skin	19
Chick-embryo skin	16
Human-skin-fibroblast medium	3.5
Bovine-aortic-smooth-muscle-cell medium	15
Mouse-skin-3T3-fibroblast medium	17

(results not shown) and that a minor part of pure human lysyl oxidase may polymerize to a 60 000- M_r form during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Kuivaniemi *et al.*, 1984). As no enzyme activity corresponding to such polymers was found in gel filtration, and as the high- M_r material did not block the antiserum from inhibiting the enzyme activity (results not shown), the polymerization may occur in such a way that the active site becomes hidden inside the polymers.

When the crude urea extract of human skin was studied by DEAE-cellulose chromatography, four peaks of lysyl oxidase activity were found, all having the same M_r , 30 000, as determined by immunoblotting after sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis (results

not shown). As the four enzyme forms were found here to be present even in crude tissue extracts, and as their presence is unaffected by the use of several proteinase inhibitors (Kagan *et al.*, 1979), these forms are not likely to be due to any artifacts. The differences may exist at the level of some post-translational modification of the enzyme protein, but the possibility is not excluded that the multiple forms may represent products of four very similar but not identical genes that are expressed in a number of tissues.

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