A simple procedure for the isolation of protein disulphide-isomerase

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A two-step procedure is described for the purification of protein disulphide-isomerase (PDI). This procedure is based on the previous finding that the β -subunit of the prolyl 4-hydroxylase tetramer ($\alpha_2\beta_2$) is identical with PDI [Koivu, Myllylä, Helaakoski, Pihlajaniemi, Tasanen & Kivirikko (1987) *J. Biol. Chem.* **262**, 6447–6449; Pihlajaniemi, Helaakoski, Tasanen, Myllylä, Huhtala, Koivu & Kivirikko (1987) *EMBO J.* **6**, 643–649]. The procedure involves purification of the prolyl 4-hydroxylase tetramer by a simple affinity chromatography and subsequent isolation of the β -subunit from the dissociated tetramer by ion-exchange chromatography.

INTRODUCTION

Protein disulphide-isomerase (PDI, EC 5.3.4.1) catalyses the rearrangement of disulphide bonds in various proteins *in vitro* and is likely to be involved in the formation of disulphide bonds during protein synthesis. The enzyme has an M_r of about 60000, has a broad specificity for protein disulphide substrates, is widely distributed in animal tissues and is present in the microsomal fraction, probably loosely associated with the luminal surface of the endoplasmic reticulum (for reviews see Anfinsen & Scheraga, 1975; Freedman & Hillson, 1980; Freedman *et al.*, 1984; Hillson *et al.*, 1984). The enzyme has also been known as thiol:protein-disulphide oxidoreductase (EC 1.8.4.2) and glutathione:insulin transhydrogenase, but subsequently all these three activities have been demonstrated to be due to a single enzyme (Bjelland *et al.*, 1983; Lambert & Freedman, 1983). Recent comparisons of cDNA-derived amino acid sequences for rat PDI (Edman *et al.*, 1985) and the β -subunit of human prolyl 4-hydroxylase (EC 1.14.11.2), and Southern-blot analyses of human genomic DNA, have indicated that PDI and the β -subunit of prolyl 4-hydroxylase are products of the same gene (Pihlajaniemi *et al.*, 1987). Additional data demonstrate that a single polypeptide indeed acts both as PDI and the β -subunit of prolyl 4-hydroxylase (Koivu *et al.*, 1987). The β -subunit isolated from the prolyl 4-hydroxylase tetramer ($\alpha_2\beta_2$) has PDI activity similar to PDI itself, and even the β -subunit when present in the prolyl 4-hydroxylase tetramer has half of this activity (Koivu *et al.*, 1987).

PDI has been purified to homogeneity from many sources (see Carmichael *et al.*, 1977; Hillson *et al.*, 1984; Koivu & Myllylä, 1986; Kaetzel *et al.*, 1987). The procedures usually consist of homogenization, heat treatment, $(\text{NH}_4)_2\text{SO}_4$ fractionation, cation-exchange chromatography and anion-exchange chromatography. Since a very simple affinity-chromatography procedure is available for isolation of the prolyl 4-hydroxylase tetramer (Tuderman *et al.*, 1975; Kedersha & Berg, 1981) and since the β -subunit can easily be isolated from this

tetramer (Berg *et al.*, 1979), we have developed an alternative procedure for the isolation of PDI. This procedure is based on the isolation of the β -subunit from the prolyl 4-hydroxylase tetramer. An example is presented on the application of this procedure for the isolation of PDI from chick embryos, but the procedure can easily be used also for many other sources.

MATERIALS AND METHODS

Materials

RNA, RNAase (type XII-A) and poly-(L-proline) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Urea of ultrapure grade was purchased from Bethesda Research Laboratories (Neu Isenburg, Germany). Randomly cross-linked RNAase was prepared as described previously (Myllylä & Oikarinen, 1983).

Purification of PDI

PDI was purified from 100–200 15-day-old chick embryos. The embryos were homogenized and the 15000 g supernatant fraction was prepared as described for the purification of prolyl 4-hydroxylase (see Kivirikko & Myllylä, 1982). This supernatant was passed through an affinity column (1.5 cm $\times$ 30 cm) containing poly-(L-proline) linked to agarose. The affinity chromatography and the elution of the tetrameric prolyl 4-hydroxylase from the column were also performed as described previously (Tuderman *et al.*, 1975; Kivirikko & Myllylä, 1982). Urea, NaCl and dithiothreitol were added to the eluate to give final concentrations of 8 M, 0.15 M and 10 mM respectively, and the tetrameric prolyl 4-hydroxylase was dissociated into its subunits by incubation at 37 °C for 30 min. The sample was then applied to a DEAE-cellulose (DE52; Whatman) column (1.0 cm $\times$ 15 cm), equilibrated with a solution containing 0.15 M-NaCl, 10 mM-EDTA and 50 mM-Tris/HCl buffer, pH 7.8, at 4 °C. The column was washed with 5 column volumes of the above solution and eluted with a 400 ml linear gradient of 0.15–1.0 M-NaCl in the same solution. Fractions (5 ml) were collected, and those containing

Abbreviation used: PDI, protein disulphide-isomerase.

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Table 1. Comparison of two procedures for purification of PDI from 150 g of chick-embryo 15000 g supernatant protein

For details see the text. Abbreviation: PAGE, polyacrylamide-gel electrophoresis.

Procedure . . .	Present work	Koivu & Myllylä (1986)
Steps	Affinity chromatography DEAE-cellulose	(NH ₄) ₂ SO ₄ fractionation CM-Sephadex DEAE-Sephacel Gel filtration
Recovery of activity (munits)	4310	5900
Recovery of protein (mg)	1.4	2.0
Specific activity (munits/mg)	3080	2960
Purity by SDS/PAGE (%)	100	95–100

most of the PDI activity were pooled and concentrated to about 3 ml in an ultrafiltration cell (Amicon, Lexington, MA, U.S.A.) with a PM-10 membrane.

In some experiments PDI was also purified by a previously reported procedure consisting of (NH₄)₂SO₄ fractionation, CM-Sephadex cation-exchange chromatography, DEAE-Sephacel anion-exchange chromatography and gel filtration (Koivu & Myllylä, 1986).

Assays

PDI activity was assayed by a method based on measurement of the rate of regeneration of incorrectly disulphide-linked RNAase to the native active form. The extent of RNAase re-activation was determined by estimating the degradation of RNA, monitoring the change in A_{260} relative to A_{280} on a spectrophotometer in dual-wavelength mode (Ibbetson & Freedman, 1976).

The protein content of each fraction was measured throughout the purification procedures by peptide absorbance at 225 nm, with bovine serum albumin as a standard.

SDS/polyacrylamide-slab-gel electrophoresis was performed with 10% polyacrylamide as the separating gel (Weber & Osborn, 1975). The gels were stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Many previous studies have demonstrated that the β -subunit of prolyl 4-hydroxylase is found in cells in two forms: in the active prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramer and in a form not associated with the α -subunit and possessing no hydroxylase activity (McGee *et al.*, 1971; Stassen *et al.*, 1974; Kao *et al.*, 1975). This β -subunit is now known to be identical with PDI (Koivu *et al.*, 1987; Pihlajaniemi *et al.*, 1987). All previously reported procedures for the isolation of PDI are based on purification of the single-subunit form of the enzyme, with the form present in the prolyl 4-hydroxylase tetramer being discarded. The present procedure uses an opposite approach: only PDI present in the prolyl 4-hydroxylase tetramer is isolated, whereas the other form is discarded.

The procedure reported here is much simpler than the previously reported ones, as it yields pure PDI in two steps (Table 1). The enzyme is eluted from the DEAE-cellulose column as a sharp peak that is well separated from the other proteins (Fig. 1). The specific activity of PDI purified with the present procedure was found to be similar to that of the enzyme obtained with the previous procedures or even slightly higher (Table 1).

In the initial purification experiments the buffer used to elute the DEAE-cellulose column did not contain EDTA. The PDI obtained in those experiments always contained an additional protein contaminant, which was detected after separation by SDS/polyacrylamide-gel electrophoresis (Fig. 2). This additional protein had the same M_r value as an isoform of cytoplasmic actin that binds to poly-(L-proline) and co-purifies with prolyl 4-hydroxylase (Kedersha *et al.*, 1986). The possibility was considered that the actin may become bound to the β -subunit of the dissociated prolyl 4-hydroxylase tetramer and therefore cannot be separated from this subunit (i.e. PDI) by DEAE-cellulose chromatography. When 10 mM-EDTA was added to the DEAE-cellulose chromatography buffer to dissociate such a possible complex, complete purification of PDI was easily obtained (Fig. 2).

The previously reported procedure for the isolation of PDI from chick embryos (Koivu & Myllylä, 1986) may likewise give pure enzyme. However, the preparations frequently have a minor contaminant. Furthermore, PDI is often partially degraded in the previous procedures, and as a result multiple enzyme bands are found on SDS/polyacrylamide-gel electrophoresis (see Kaetzel *et al.*, 1987).

Fig. 1. Elution of PDI from DEAE-cellulose chromatography in the last step of the purification procedure

Conditions were as given in the Materials and methods section, except that the amount of starting material for the purification procedure was 11 g, and the size of the fractions was 2 ml. ●—●, A_{225} ; -----, concn. of NaCl. Fractions nos. 16–23 contained the pure enzyme.

Fig. 2. SDS/polyacrylamide-slab-gel electrophoresis of PDI isolated from chick embryos

Purification was carried out in the absence (lanes 1 and 2) and in the presence (lanes 3 and 4) of 10 mM-EDTA in the buffer of the DEAE-cellulose column chromatography (see the Materials and methods section). Protein M_r standards are indicated.

The recovery of PDI activity from chick-embryo 15000 g supernatant with the present procedure was 73 % of that obtained with the previous procedure (Table 1). As all the purification procedures omit one of the two forms of PDI present in cells, the recovery of activity units is in part dependent on the ratio between the two enzyme forms in the tissue in question. These ratios generally favour the existing procedures, as in most cells and tissues less than half of the total β -subunit protein, measured by radioimmunoassays, is present in the prolyl 4-hydroxylase tetramer (for references see Kivirikko & Myllylä, 1980). Cells or tissues in which about half or more than half of the total PDI protein is found in the $\alpha_2\beta_2$ tetramer include freshly isolated chick-embryo tendon and cartilage cells, and several chick-embryo tissues between days 10 and 17 of development. The percentage distribution of total PDI between the two cellular forms is currently known only for a few tissues, but the percentage of the tetramer form appears to be highest in cells and tissues actively synthesizing collagen (see Kivirikko & Myllylä, 1980). It may be noted that, because the present procedure is very simple and yields pure enzyme, this procedure may be preferred even in cases where the percentage of PDI in the tetramer form is likely to be low. For example, in the case of human placental tissue, 5 mg of PDI can be isolated from ten placentas by using a previous procedure (Koivu & Myllylä, 1986) whereas the present procedure yields only about 1 mg. Nevertheless, the previous procedure is much more tedious, and the purity of the final enzyme is

after only about 90 %. Accordingly, the present procedure may be preferred in such situations.

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