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Percoll Reversibly Inhibits Superoxide Dismutase

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Incubation of pea leaf extracts (*Pisum sativum* L.) at 6°C in isoosmotic media containing different Percoll concentrations significantly represses the total superoxide dismutase (SOD) activity in a concentration- and time-dependent manner. After 24 h incubation at 6°C, 30-45 % Percoll concentrations bring about an inhibition of Mn-SOD activity of more than 50 %. Isozyme Cu,Zn-SOD II is affected to a lesser extent, with a maximum inhibition of 36 % at high Percoll concentrations, whereas isozyme Cu,Zn-SOD I undergoes only slight variations. However, dilution of the samples followed by electrophoresis completely removes the Percoll inhibitory action. Results suggest that superoxide dismutases could be adsorbed onto the Percoll surface through electrostatic interactions.

Key words: Cell organelles, Percoll inhibition, *Pisum sativum* L., Superoxide dismutases.

Percoll consists of colloidal silica particles of 15-30 nm diameter coated with polyvinylpyrrolidone, and is a widely used gradient medium for density-gradient centrifugation of cells and subcellular organelles (13). In plants, Percoll media have been widely used for the isolation of chloroplasts (11, 13, 14, 19), mitochondria (7, 12, 13) and peroxisomes (10, 15), and also for the purification of protoplasts (11). Percoll has been reported to be non-toxic to cells (13), but recently WAKEFIELD *et al.* (20) have demonstrated the existence of an interaction between Percoll particles and the cell surface of mice macrophages which produces inhibitory effects on cell adherence. Although Percoll is very convenient for the separation of cell organelles under isoosmotic conditions, undesirable effects of Percoll on glyoxysomes (15) and functionality of chloroplasts (18) have been reported. Moreover, adverse and rapid effects of Percoll on

Abbreviations: SOD, superoxide dismutase. Mn-SOD, manganese-containing superoxide dismutase. Cu/Zn-SOD, copperzinc-containing superoxide dismutase. NBT, nitroblue tetrazolium.

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the intactness of peroxisomes isolated from green leaf tissues when stored in the Percoll gradient medium have also been described (10, 15).

To our knowledge, no evidence has been presented on the toxicity of Percoll towards enzymatic activities thus far, and apparently many enzyme assays can be carried out in the presence of Percoll without interferences (13). However, in the course of recent experiments carried out in our laboratory on the subcellular localization of superoxide dismutases (EC 1.15.1.1) (5) in *Pisum sativum* L. leaves using discontinuous density-gradients of Percoll (5-45 %), considerable losses in SOD activity were found in the cell fractions obtained. This presented serious difficulties in the assignment of isozyme activity to specific cell components. Accordingly, a study was conducted on the effect of different Percoll concentrations at low temperature on the activity of Mn- and Cu,Zn-containing superoxide dismutases, using as a model those present in *Pisum sativum* L. leaves which have been thoroughly studied in our laboratory (1, 2, 6, 9, 16).

Materials and Methods

Pea seeds (*Pisum sativum* L., var. Lincoln) were germinated and grown in perlite in a growth chamber, Conviron PGW-36, under the conditions described elsewhere (2). Leaves were harvested 18 to 25 days after planting. Extracts from pea leaves were prepared in isoosmotic media similar to those described for the isolation of plant organelles by Percoll density-gradient centrifugation (15), but containing 0.2 % Triton X-100 and the following different Percoll (Sigma) concentrations (%): 5, 15, 30, and 45 (v/v), respectively. The basic medium had the following composition: 25 mM Hepes-KOH buffer, pH 7.5, containing 0.5 M sucrose, 0.1 % bovine serum albumin and 0.2 % Triton X-100.

Leaf samples were homogenised on ice in media containing 0 % (control), 5, 15, 30, and 45 % Percoll (v/v), respectively, using a Sorvall Omnimixer (leaf to medium ratio 1:2; w/v). Homogenates were filtered through four layers of nylon cloth and centrifuged at 12,000 *g* for 10 min. The resulting supernatants were immediately incubated at 6°C (zero time), and aliquots were taken at different time intervals and assayed for SOD activity.

Total SOD activity of pea leaf extracts containing different Percoll concentrations was determined by the ability of SOD to inhibit nitrite formation from hydroxylamine by the $O_2^{\cdot-}$-generating system, xanthine oxidase plus xanthine (4), with the following modifications: sulphanilic acid and α-naphthylamine were replaced by identical concentrations of sulphanilamide and naphthylethylene diamine diHCl, respectively. SOD isozymes in the Percoll incubation mixtures (Mn-SOD, Cu/Zn-SOD I and Cu/Zn-SOD II) were individualized by electrophoresis on thin-layer polyacrylamide gels at 10 % gel concentrations with an LKB Multiphor system equipped with a cryothermostat. SODs were localized on the gels by the method of NBT reduction by $O_2^{\cdot-}$ radicals generated photochemically (21). Blanks of the media used were run on parallel gels and no interference by Percoll in the SOD detection method was found. The isozyme activity was quantitated by recording the transmittance of the thin-layer gels in a Vernon PHI-6 densitometer and calculating the areas under the transmittance peaks by cutting out and weighing the peaks drawn by the densitometer.

Results and Discussion

The total superoxide dismutase activity of the Percoll-containing pea extracts at different incubation times is shown in figure 1. The range of Percoll concentra-

tions used (5-45 %; v/v) is representative of moderate concentrations employed for the isolation of plant subcellular particles (7, 12, 13, 15) by density-gradient centrifugation, although concentrations as high as 70-90 % have also been reported (10, 11, 13, 19). Percoll inhibits SOD activity and this inhibition is both concentration- and time-dependent. Even at the zero time, a 45 % Percoll concentration brought about a statistically significant inhibition of SOD activity ($p<0.05$) as compared with controls. After 4 h incubation, the activity repression was more significant at 30 % and 45 % Percoll ($p < 0.01$), and the inhibitory effect was considerably enhanced after 24 h, with activity losses at those Percoll concentrations statistically significant at $p < 0.001$ as compared with controls. Even after 7 days incubation, when the activity of controls had significantly decayed, probably due to endogenous inactivation by extract products, the repressive effect of increasing Percoll concentrations was still evident.

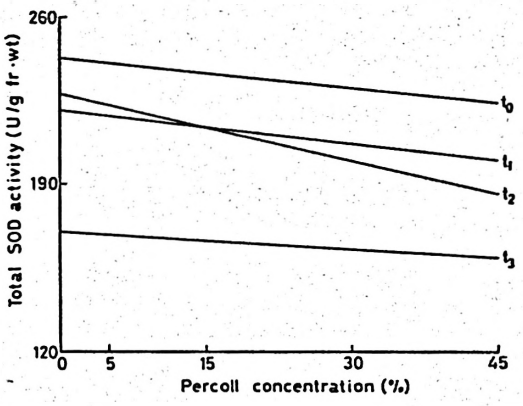

Fig. 1. *Total superoxide dismutase activity of pea leaf extracts containing different Percoll concentrations and incubated at 6°C.* Two incubation mixtures from different leaf batches were prepared for each Percoll concentration and controls. Aliquots were withdrawn at time intervals and assayed for SOD activity in triplicate. Each plot represents the regression line of total SOD activity versus different Percoll concentrations at incubation times of 0 h (t_0), 4 h (t_1), 24 h (t_2) and 7 days (t_3).

Pea leaves contain three electrophoretically distinct superoxide dismutases, a slower moving Mn-containing SOD and two copper zinc-containing SODs, named I and II in order of increasing mobility (1). The three isoenzymes have been previously purified and fully characterized (3, 16). After 24 h incubation at 6°C, 30 % and 45 % Percoll concentrations brought about an inhibition of Mn-SOD activity of more than 50 % (fig. 2). Cu,Zn-SOD isozyme II was affected to a lesser extent by Percoll, with a maximum inhibition of 36 % at high Percoll concentrations, whereas Cu,Zn-SOD isozyme I underwent only a slight change. The effect of diluting samples prior to electrophoresis, to a final Percoll concentrations in the range 1-9 % is shown in figure 3. When incubation mixtures were diluted with 50 mM phosphate buffer, pH 7.8, prior to electrophoresis and subjected to an identical procedure, the inhibition of Mn-SOD by Percoll was reversed with increasing dilutions, and after a 5-fold dilution, when the Percoll concentration in the incubation mixture was 1-9 %, all the samples had identical activities compared to the control. Thus, dilution of the sample clearly led to the removal of the inhibitory effect of Percoll.

The reversibility of SOD inhibition by Percoll is not due to a simple dilution of the samples. As is shown in figure 1, samples for total SOD activity determination were about 400-fold diluted in the final reaction mixture, but the repressive effect of Percoll on enzymatic activity was evident. However, dilution of samples followed by electrophoresis completely removed the Percoll inhibitory action (fig. 3). These results suggest that Mn-SOD and Cu,Zn-SOD II may be adsorbed onto the Percoll surface possibly through weak Van der Waals-type

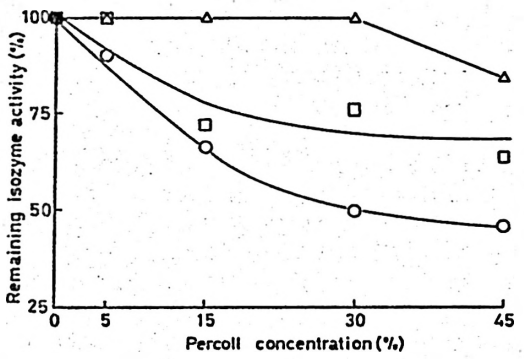

Fig. 2. *Effect of Percoll on superoxide dismutase isozymes after 24 h incubation at 6°C.* Incubation media of pea leaf extracts without Percoll (controls) and with 5-45 % Percoll concentrations were prepared in duplicate and incubated at 6°C. After 24 h, samples were subjected to polyacrylamide gel electrophoresis and stained for SOD activity by a photochemical method. The relative isozyme activities (in cm^2) were calculated by densitometry and integration of the activity areas obtained. Each point represents the mean of two different samples. O, Mn-SOD; Δ, Cu/Zn-SOD I; □, Cu/Zn-SOD II.

interactions, and/or hydrogen bonding between oxygen or hydroxyl groups of colloidal silica and certain residues of amino acids on the protein surface, such as amino and carboxyl groups (8). These adsorption processes may thus hinder access of substrate (O_2^- radicals) into the SOD active center. However, the fact that electrophoresis of appropriately diluted Percoll-containing mixtures fully restores the SOD activity seems to point out that the application of an electric field produces a weakening of the interaction between the proteins and Percoll leading to a reversal of the adsorption process.

The different behaviour of the three SOD isozymes towards Percoll may be a reflection not only of the structural differences existing between Mn-SODs and Cu,Zn-SODs (17), but also between the two pea leaf Cu,Zn-SODs (I and II). It has recently been demonstrated by Duke and Salin (3) that significant discrepancies exist in the molecular properties of the two Cu,Zn-isozymes (I and II), such as net charge, absorption spectra, subunit size and amino acid composition.

The interaction found between Percoll and the metalloenzyme family of superoxide dismutase raises interesting questions from the viewpoint of the physical chemistry of surface effects between colloidal silica particles and enzymes, as well as those related to the actual kinetic mechanism of action of superoxide dismutases. In addition, it also indicates that special caution should be exercised in studies on superoxide dismutases at subcellular level using Percoll density-gradient centrifugation for the isolation of cell fractions. Assays of total SOD activity should be carried out immediately after cell disruption and if possible, Percoll should be quickly removed from subcellular fractions. At the same time, in determining the activity of individual superoxide dismutase isozymes in cell fractions by gel electrophoresis, appropriate dilutions of samples should be made, provided the SOD levels remain within detection limits, in order to re-

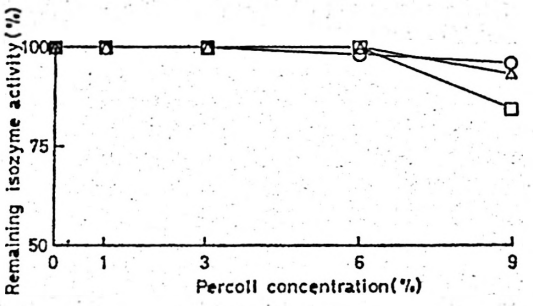

Fig. 3. *Effect of diluting 24 h incubation mixtures containing 5-45 % Percoll on superoxide dismutase isozyme activity.* Conditions as described for fig. 2, except that samples incubated for 24 h at 6°C were 5-fold diluted with 50 mM phosphate buffer, pH 7.8, immediately prior to electrophoresis, so that the final Percoll concentrations in electrophoresed samples were 1-9 %.

verse the Percoll inhibitory action. Otherwise, false conclusions could be drawn regarding the intracellular localization of superoxide dismutase isozymes.

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Resumen

La incubación de extractos de hojas de guisante (*Pisum sativum*, L.) a 6°C en medios isoosmóticos conteniendo diferentes concentraciones de Percoll inhibe significativamente la actividad total superóxido dismutasa (SOD) en función del tiempo y de la concentración del producto. Después de 24 horas de incubación a 6°C, concentraciones del 30-45 % de Percoll producen una inhibición de la actividad Mn-SOD de más de un 50 %. La isoenzima Cu,Zn-SOD II resulta afectada en menor grado, con una inhibición máxima de un 36 %, mientras que la isoenzima Cu,Zn-SOD I apenas experimenta variaciones. La dilución de las muestras y la posterior electroforesis de las mismas, elimina completamente la acción inhibitoria del Percoll. Los resultados obtenidos sugieren que las superóxido dismutasas podrían ser adsorbidas sobre la superficie del Percoll mediante interacciones electrostáticas.

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