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Outer envelope preparation

The procedure described previously (Seedorf et al., 1995) was optimized as follows: 5,000 *g* pea leaves were harvested and minced in 15 liter of buffer A (20 mM Mops, 13 mM Tris, 0.1 mM $MgCl_2$, 330 mM sorbitol, 0.05% BSA, 0.1 mM PMSF, 2 mM β-mercaptoethanol, pH 7.9) using a Waring blender. The cell fragments were removed by passing the solution through four layers of cheese cloth and one layer of 25 μm gaze. The suspension was pelleted at 1,500 *g* for 5 min at 4°C. The supernatant was centrifuged again for 5 min at 1,500 *g* at 4°C, and both pellets were thoroughly resuspended in buffer A, combined and adjusted to 680 ml. The suspension was layered on top of 34 Percoll gradients formed by 12 ml of 40% and 7 ml of 80% Percoll in 330 mM sorbitol, 50 mM Mops, 0.1 mM PMSF, 2 mM β-mercaptoethanol, pH 7.9, and centrifuged for 10 min at 5,000 *g*. Chloroplasts on top of the 80% Percoll layer were combined and diluted to 1 liter using 330 mM sorbitol, 0.1 mM PMSF, 2 mM β-mercaptoethanol, pH 7.6. Chloroplasts were repelleted at 2,250 *g* for 5 min at 4°C. The pellet was resuspended again in 1 liter of buffer, and the process was repeated. Pellets were resuspended to a final volume of 240 ml in 0.65 M sucrose, 10 mM Tricine, 1 mM EDTA, 0.1 mM PMSF, 2 mM β-mercaptoethanol, pH 7.9, and kept on ice for 10 min. Chloroplasts were ruptured by 50 strokes in a Dounce homogenizer. The volume was then adjusted to 720 ml by slow addition of buffer B (10 mM Tricine, 1 mM EDTA, 0.1 mM PMSF, 2 mM β-mercaptoethanol, pH 7.9), and the suspension was centrifuged for 10 min at 4,000 *g* at 4°C. The volume of supernatant was adjusted again using buffer B to 720 ml and centrifuged for 30 min at 30,000 *g* and 60 min at 150,000 *g* at 4°C. The pellet was carefully washed to resuspend the envelopes present on top of the thylakoids. The envelope suspension was diluted to 120 ml using buffer B, and 10-ml fractions were layered on top of a sucrose step gradient (8 ml 0.465 M sucrose, 10 ml 0.8 M sucrose, and 8 ml 0.996 M sucrose in buffer C [10 mM sodium phosphate, 1 mM EDTA, 2 mM β-mercaptoethanol, pH 7.9]). The gradients were centrifuged for 3 h at 100,000 *g* at 4°C. The layer on top of the 0.8 M sucrose layer contained the outer envelope. The outer envelope was collected, diluted three times using buffer C, and pelleted by centrifugation for 1 h at 100,000 *g* at 4°C. The pellet was resuspended in 3 ml of buffer C, directly frozen in liquid nitrogen, and stored at −80°C for further use. The amount of the outer envelope membranes was determined using the Bio-Rad Laboratories assay.