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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF ENGINEERING & TECHNOLOGY

LT28:Application of centrifugation for preparative and analytical purposes

Content Outline

1. Practical application of Preparative centrifuge
2. Application of differential centrifuge
3. Affinity purificat of membrane vesicles
4. Application of analytical centrifugation

Application of centrifugation for preparative and analytical purposes

Practical applications of preparative centrifugation

In this section, the practical methods, application and protocol for use of differential centrifugation, density gradient ultracentrifugation and affinity methodology will be described. The isolation of following components will be described:

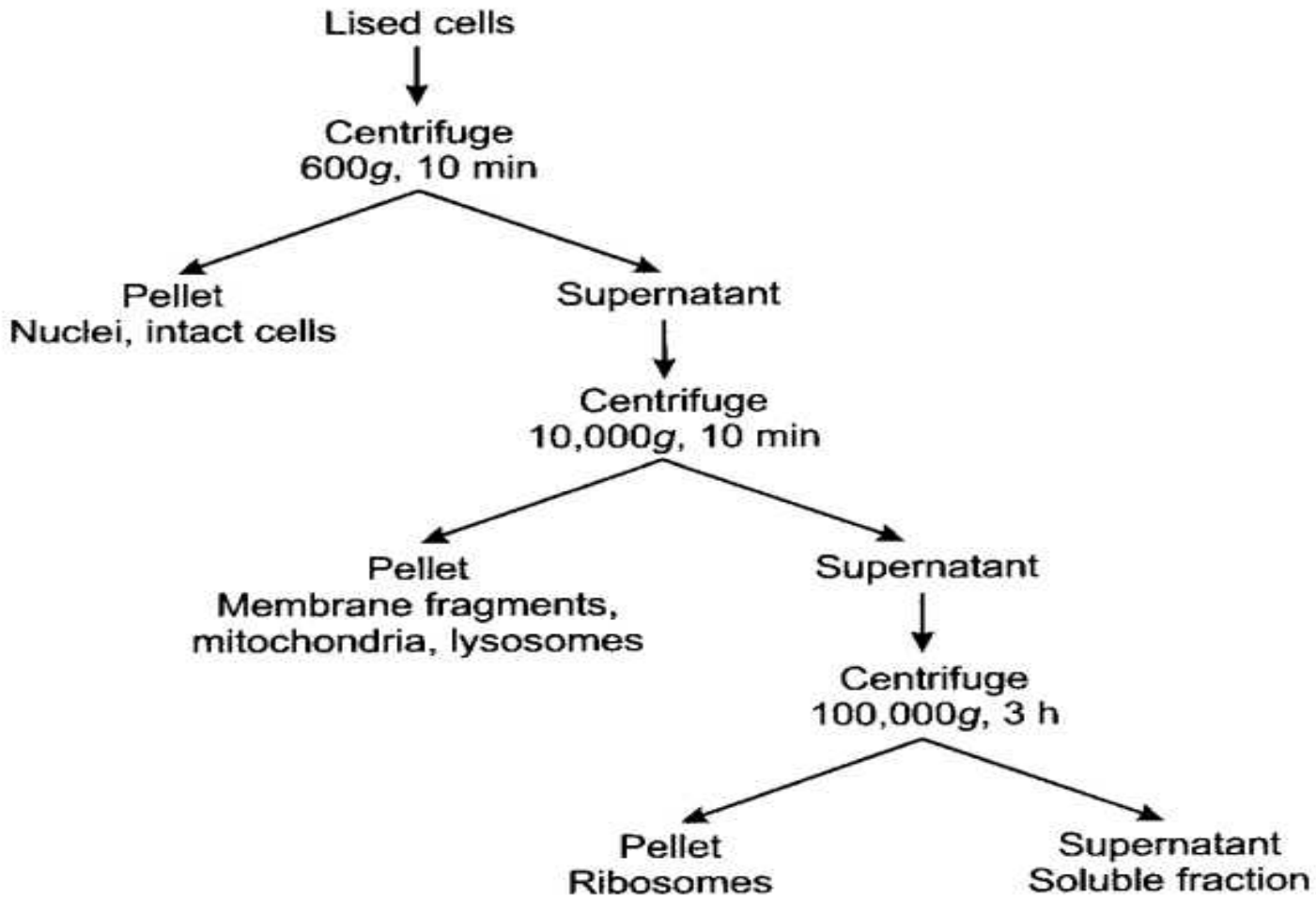
The isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density.

The isolation of highly purified sarcolemma vesicles outlined

The subfractionation of liver mitochondrial membrane

Differential centrifugation

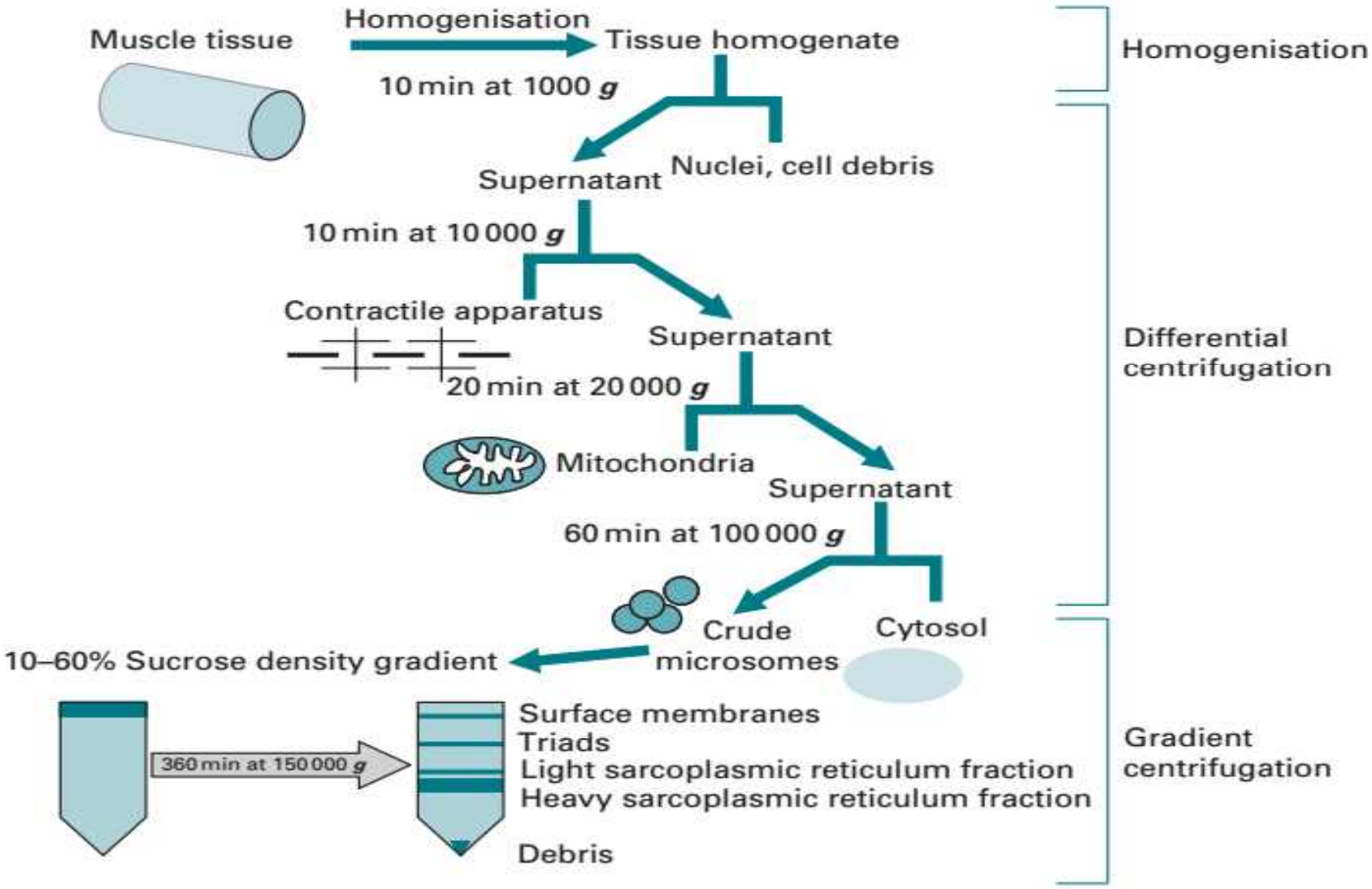
1. Differential centrifugation is very useful for the separation of subcellular organelles and membranes. The starting material is usually a tissue homogenate or lysed cells. For the optimum homogenisation of tissue specimens, mincing of tissue has to be performed in the presence of a biological buffer system that exhibits the right pH value, salt concentration, stabilising co-factors and chelating agents. Proteae inhibitors are also added to minimize degradation of subcellular fractions by endogeneos enzymes. The standard procedure is illustrated below



Typical differential centrifugation protocol. In order to obtain more purified fractions, the pellets could be resuspended in buffer and recentrifuged.

Subcellular fractionation

For subcellular fractionation a combined protocol of differential centrifugation followed by density gradient centrifugation. Depending on the amount of starting material, which would usually range between 1 and 500 g in the case of skeletal muscle preparations, a particular type of rotor and size of centrifuge tubes is chosen for individual stages of the isolation procedure. The repeated centrifugation at progressively higher speeds and longer centrifugation periods will divide the muscle homogenate into distinct fractions. Typical values for centrifugation steps are 10 min for 1000 g to pellet nuclei and cellular debris, 10 min for 10 000 g to pellet the contractile apparatus, 20 min at 20 000 g to pellet a fraction enriched in mitochondria, and 1 h at 100 000 g to separate the microsomal and cytosolic fractions. Mild salt washes can be carried out to remove myosin contamination of membrane preparations. Sucrose gradient centrifugation is then used to further separate microsomal subfractions derived from different muscle membranes. A combined protocol has been described below:


Muscle tissue
Homogenisation
Tissue homogenate
10 min at 1000 g
Nuclei, cell debris
Supernatant
10 min at 10 000 g
Contractile apparatus
Supernatant
20 min at 20 000 g
Mitochondria
Supernatant
60 min at 100 000 g
Crude microsomes
Cytosol
10–60% Sucrose density gradient
360 min at 150 000 g
Surface membranes
Triads
Light sarcoplasmic reticulum fraction
Heavy sarcoplasmic reticulum fraction
Debris
Homogenisation
Differential centrifugation
Gradient centrifugation

Affinity purification of membrane vesicles

Membrane vesicles can be separated by using lectins. Lectins are plant proteins that bind tightly to specific carbohydrate structures. These lectins only binds to sarcolemma vesicles but leave aside vesicles derived from the transverse tubules. Therefore only sarcolemma vesicles are agglutinated by the wheat germ lectin and the aggregate can be separated from the transverse tubular fraction by centrifugation for 2 min at 15 000 g. The electron microscopical characterisation of agglutinated surface membranes revealed large smooth sarcolemma vesicles that had electron-dense entrapments.

To remove these vesicular contaminants, originally derived from the sarcoplasmic reticulum, immobilised surface vesicles are treated with low concentrations of the non-ionic detergent Triton X-100. This procedure does not solubilise

To remove these vesicular contaminants, originally derived from the sarcoplasmic reticulum, immobilised surface vesicles are treated with low concentrations of the non-ionic detergent Triton X-100. This procedure does not solubilise integral membrane proteins, but introduces openings in the sarcolemma vesicles for the release of the much smaller sarcoplasmic reticulum vesicles. To remove the lectin from the purified vesicles, the fraction is incubated with the competitive sugar N-acetylglucosamine that eliminates the bonds between the surface glycoproteins and the lectin. A final centrifugation step for 20 min at 150 000 g results in a pellet of highly purified sarcolemma vesicles. A quick and convenient analytical method of confirming whether this subcellular fractionation procedure has resulted in the isolation of the muscle plasmalemma is immunoblotting with a mini electrophoresis unit. Using antibodies to markers of the transverse tubules and the sarcolemma, such as the a1S-subunit of the dihydropyridine receptor of 170 kDa and dystrophin of 427 kDa, respectively, the separation of both membrane species can be monitored.

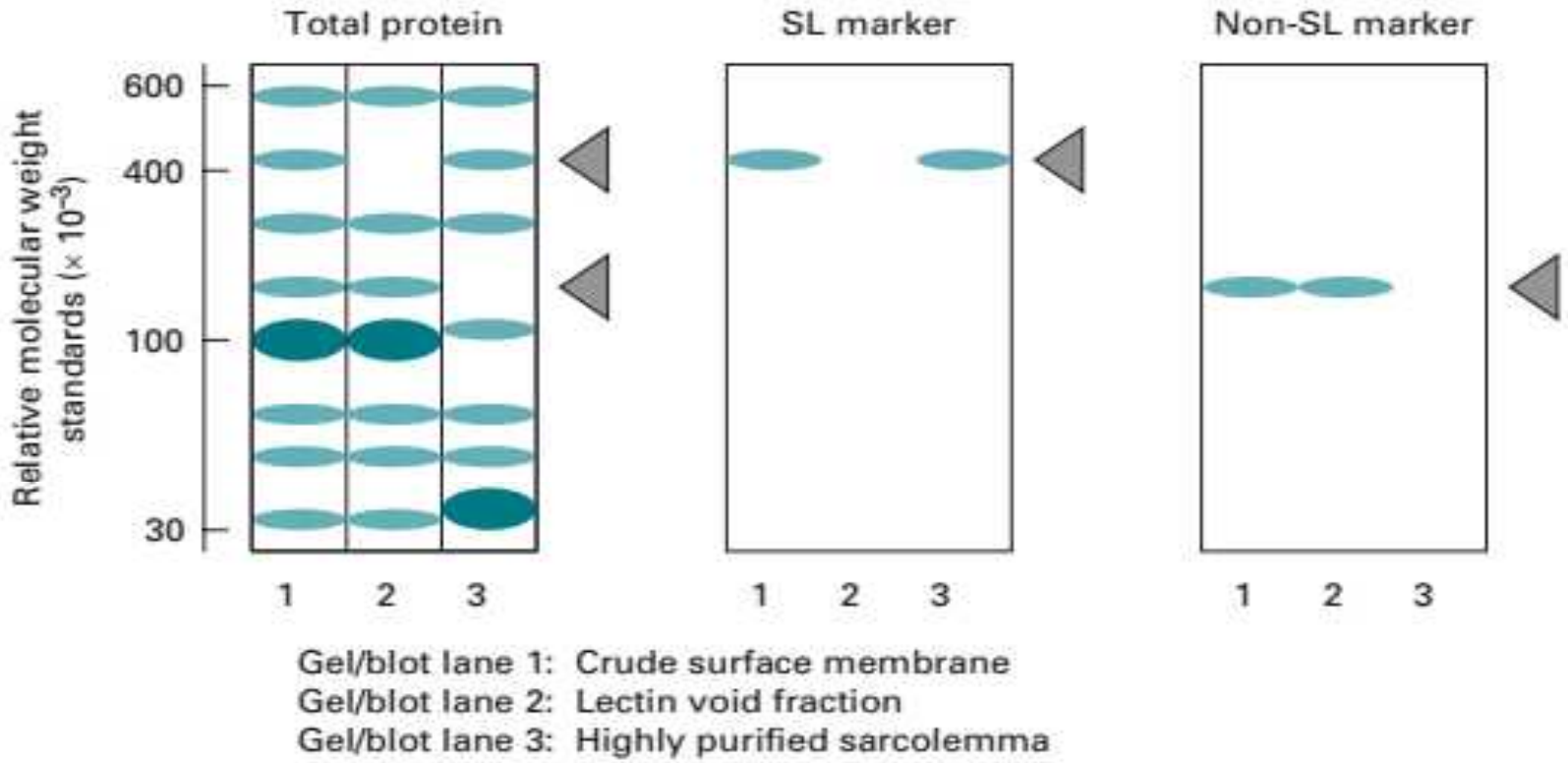


Diagram of the immunoblot analysis of this subcellular fractionation procedure (b). The sarcolemma (SL) and non-SL markers are surface-associated dystrophin of 427 kDa and the transverse-tubular a1S-subunit of the dihydropyridine receptor of 170 kDa, respectively.

Application of Analytical centrifugation

Determination of molecular shape, size and mass

The sedimentation velocity method can be employed to estimate sample purity. Sedimentation patterns can be obtained using the Schlieren optical system. This method measures the refractive index gradient at each point in the ultracentrifugation cell at varying time intervals. During the entire duration of the sedimentation velocity analysis, a homogeneous preparation forms a single sharp symmetrical sedimenting boundary. Such a result demonstrates that the biological macromolecules analysed exhibit the same molecular mass, shape and size.

Relative molecular mass determination

•For the accurate determination of the molecular mass of solutes in their native state, analytical ultracentrifugation represents an unrivalled technique. The method requires only small sample sizes (20–120 mm3) and low particle concentrations (0.01–1 g dm3) and biological molecules with a wide range of molecular masses can be characterized

•At the start of an experiment using the boundary sedimentation method, the biological particles are uniformly distributed throughout the solution in the analytical cell. The application of a centrifugal field then causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the centre of rotation.

•The solvent that has been cleared of particles and the solvent still containing the sedimenting material form a sharp boundary. The movement of the boundary with time is a measure of the rate of sedimentation of the biomolecules.

•The sedimentation coefficient depends directly on the mass of the biological particle. The concentration distribution is dependent on the buoyant molecular mass. The movement of biomolecules in a centrifugal field can be determined and a plot of the natural logarithm of the solute concentration versus the squared radial distance from the centre of rotation (ln c vs. r2) yields a straight line with a slope proportional to the monomer molecular mass.

Test your understanding

Lectins are

a. Carbohydrate binding plant proteins
b. Carbohydrate binding animal origin protein
c. protein binding plant protein
d. none of the above

To separate particles of same density, what solution wil you recommend

a. Isopycnic density gradient centrifugation
b. Labelling with H3 or C14 to increase the mass and hence density
c. Differential centrifugation
d. Rate zonal centrifugation

During separation of membrane vesicles using centrifugation, lectins only binds to

a. sarcolemma vesicles
b. vesicles derived from the transverse tubules
c. Both (a) and (b)
d. None of the above

Disc stack centrifuge is

a. Continuous flow centrifuge
b. Batch centrifuge
c. Semi-Continuous

One Svedberg unit equals 10–13 s.

a. 10-10 s
b. 10–13 s
c. 10-6 s

References & Further reading

1. Wilson, K, Walker, J., Principles and Techniques of Practical Biochemistry. 5th Ed. - Cambridge University Press,. Cambridge 1999.
2. Biotechniques, Theory & Practice: Second Edition by SVS Rana, Rustogi Publications.
3. Biochemical Methods of Analysis, Saroj Dua And Neera Garg : Narosa Publishing House, New Delhi.
4. Bioanalytical Techniques, M.L. Srivastava, Narosa Publishing House, New Delhi.

This analytical method is especially useful for the characterisation of membrane vesicles, when no simple and fast assay systems for testing marker enzyme activities are available.