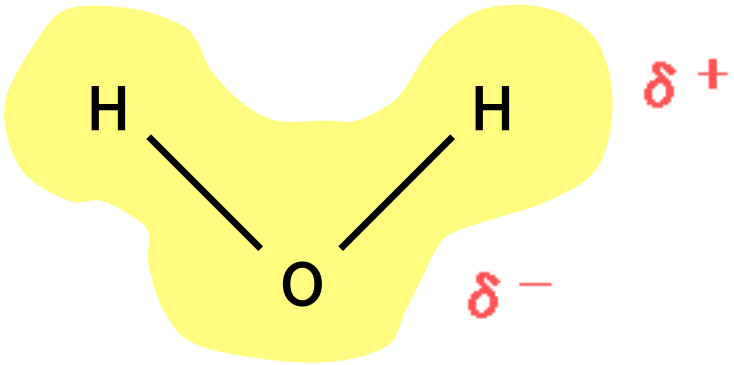
HPLC and Hydrophobic Interaction Chromatography by Dr. Yaj B.

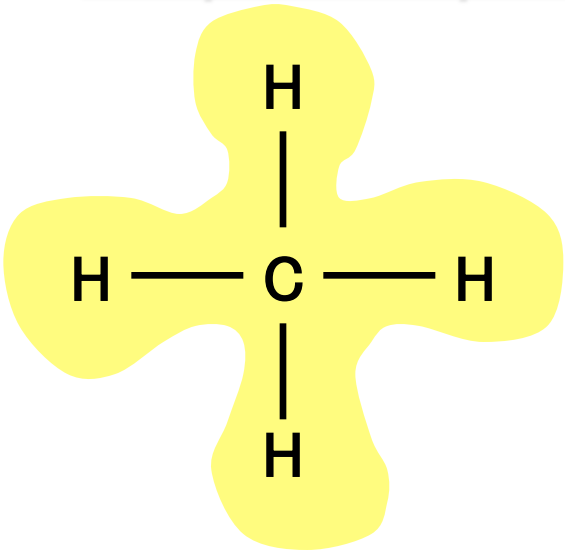
HPLC separation mode

- **Normal phase chromatography (NP)**
- **Reversed phase chromatography (RP)**
- **Size exclusion chromatography (SEC)**
- **Ion exchange chromatography (IEX)**
- **Affinity chromatography**

Polar compound



Non-polar compound



- **Bonding electrons are not shared evenly.**
- **The end of the bond with electrons becomes partially negative.**
- **The end of the bond without electrons becomes partially positive.**

Polar compounds are soluble in polar solvents.
Non-polar compounds are soluble in non-polar solvents.

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What is normal-phase HPLC (NP-HPLC) ?

Normal-phase HPLC (NP-HPLC), or adsorption chromatography, separates analytes based on adsorption to a stationary surface and by polarity

Normal Phase Chromatography

Interaction : **Adsorption**

Packing materials : **Polar ex.** **Silica gel**
Silica-NH2
Silica-CN
Silica-OH

Mobile phase : **Non-polar** **ex.** **n-Hex/CH2CL2**
iso-Oct/IPA
iso-Oct/AcOEt

Sample : **Fat-soluble**
Different polarity

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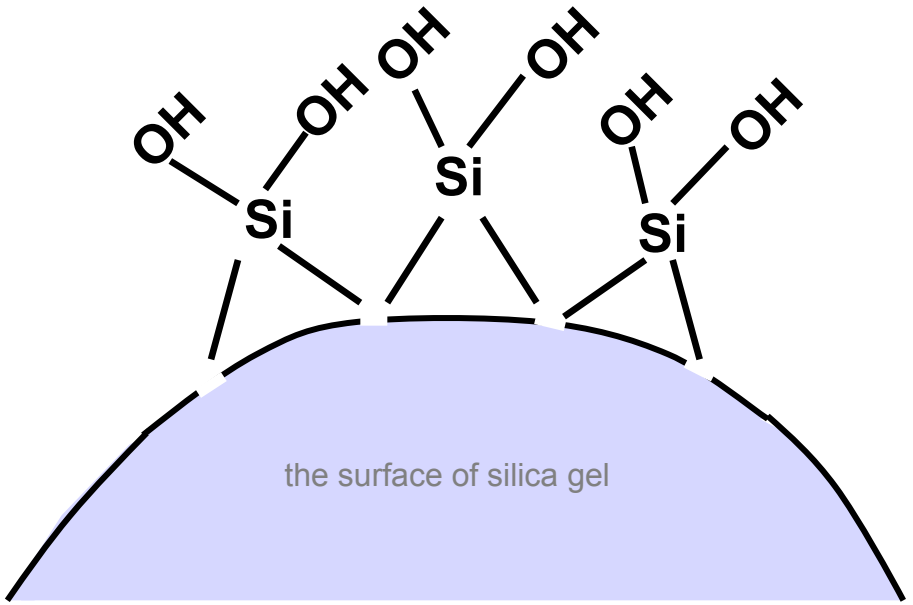
Normal Phase Chromatography

Packing material

- Popular packing material is silica gel.
- Silanol radicals (-Si-OH) on the surface of silica gel act as the active site and the sample is separated.



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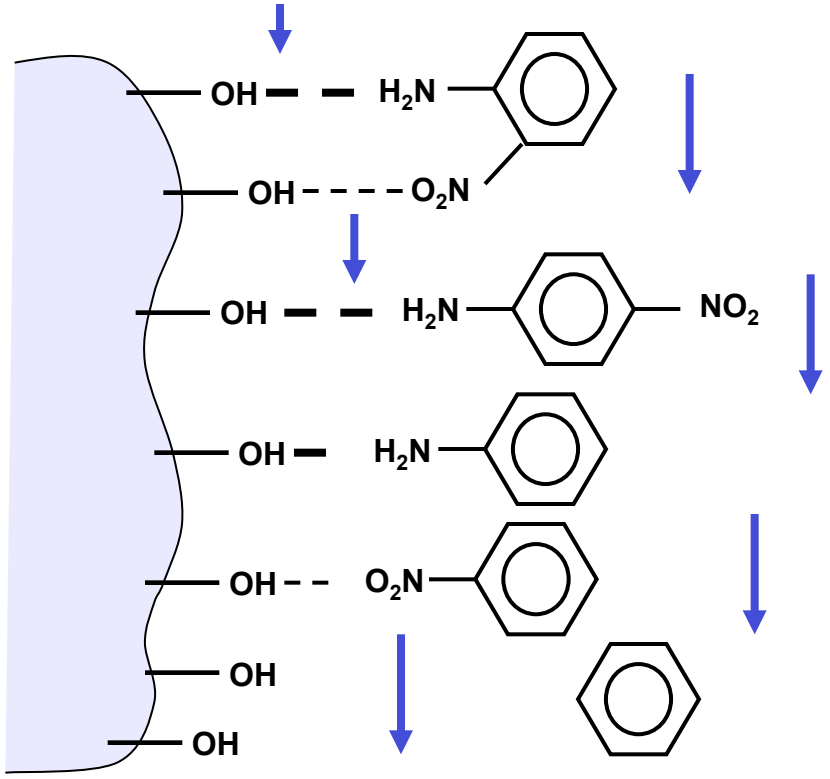
Normal Phase Chromatography

Interaction



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Normal Phase Chromatography

Mobile phase solvents

n-Hexane	(n-Hex)
iso-Octane	(iso-Oct)
Chloroform	($CHCl_3$)
Dichloromethane	(CH_2Cl_2)
Ethylacetate	(AcOEt)
Isopropylalchol	(IPA)
Tetrahydrofran	(THF)
Dioxane	
Acetonitrile	(CH_3CN)
Ethanol	(EtOH)
Methanol	(MeOH)
Amines	
Acids	

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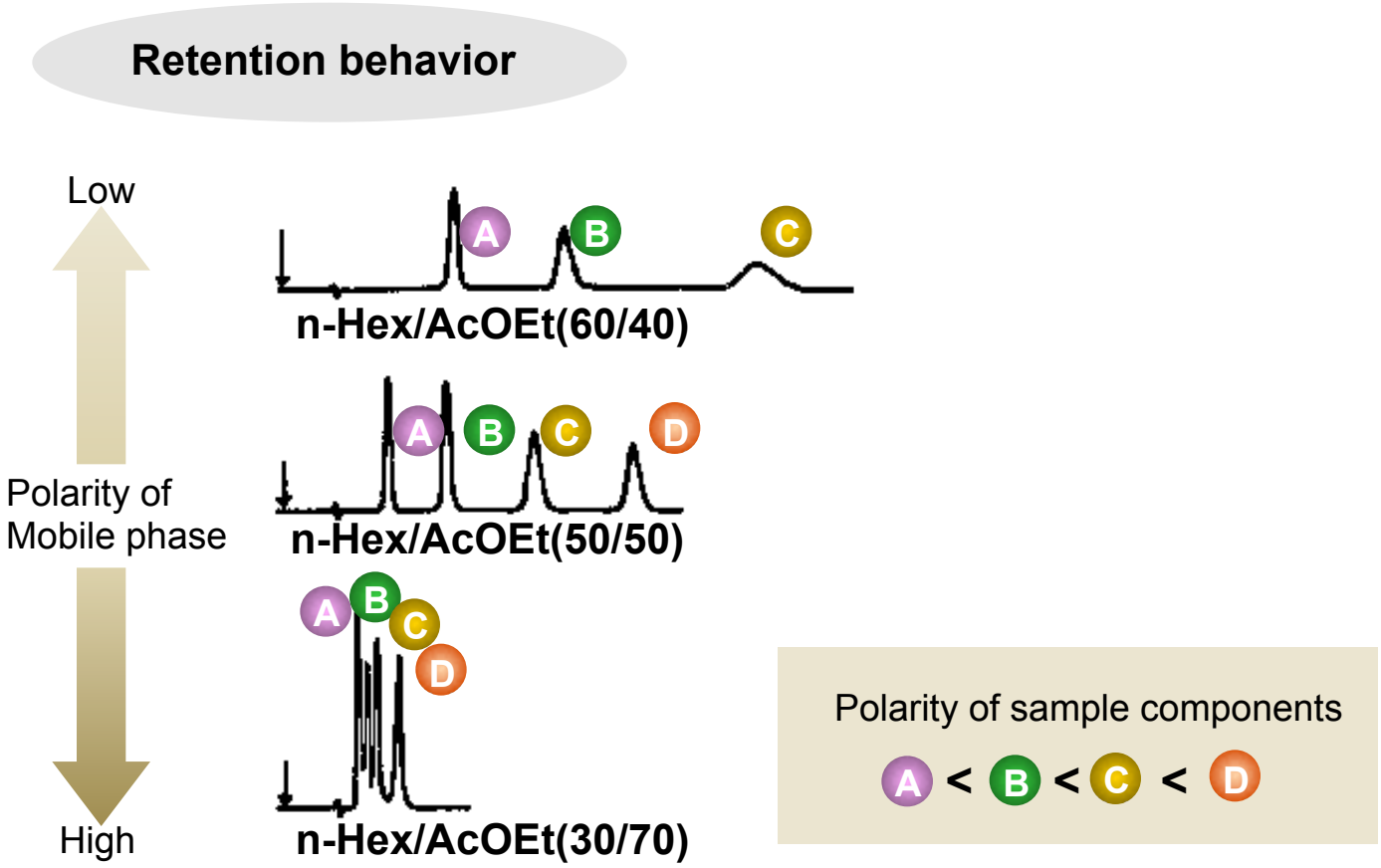
Normal Phase Chromatography



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Normal Phase Chromatography

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Separation of cellular nonpolar neutral lipids by normal-phase chromatography and analysis by electrospray ionization mass spectrometry

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Abstract

Neutral lipids are an important class of hydrophobic compounds found in all cells that play critical roles from energy storage to signal transduction. Several distinct structural families make up this class, and within each family there are numbers of individual molecular species. A solvent extraction protocol has been developed to efficiently isolate neutral lipids without complete extraction of more polar phospholipids. Normal-phase HPLC was used for the separation of cholesteryl esters (CEs), monoalkylether diacylglycerols, triacylglycerols, and diacylglycerols in a single HPLC run from this extract. Furthermore, minor lipids such as ubiquinone-9 could be detected in RAW 264.7 cells. Molecular species that make up each neutral lipid class can be analyzed both qualitatively and quantitatively by on-line LC-MS and LC-MS/MS strategies. The quantitation of >20 CE molecular species revealed that challenging RAW 264.7 cells with a Toll-like receptor 4 agonist caused a >20-fold increase in the content of CEs within cells, particularly those CE molecular species that contained saturated (14:0, 16:0, and 18:1) fatty acyl groups. Longer chain CE molecular species did not change in response to the activation of these cells.

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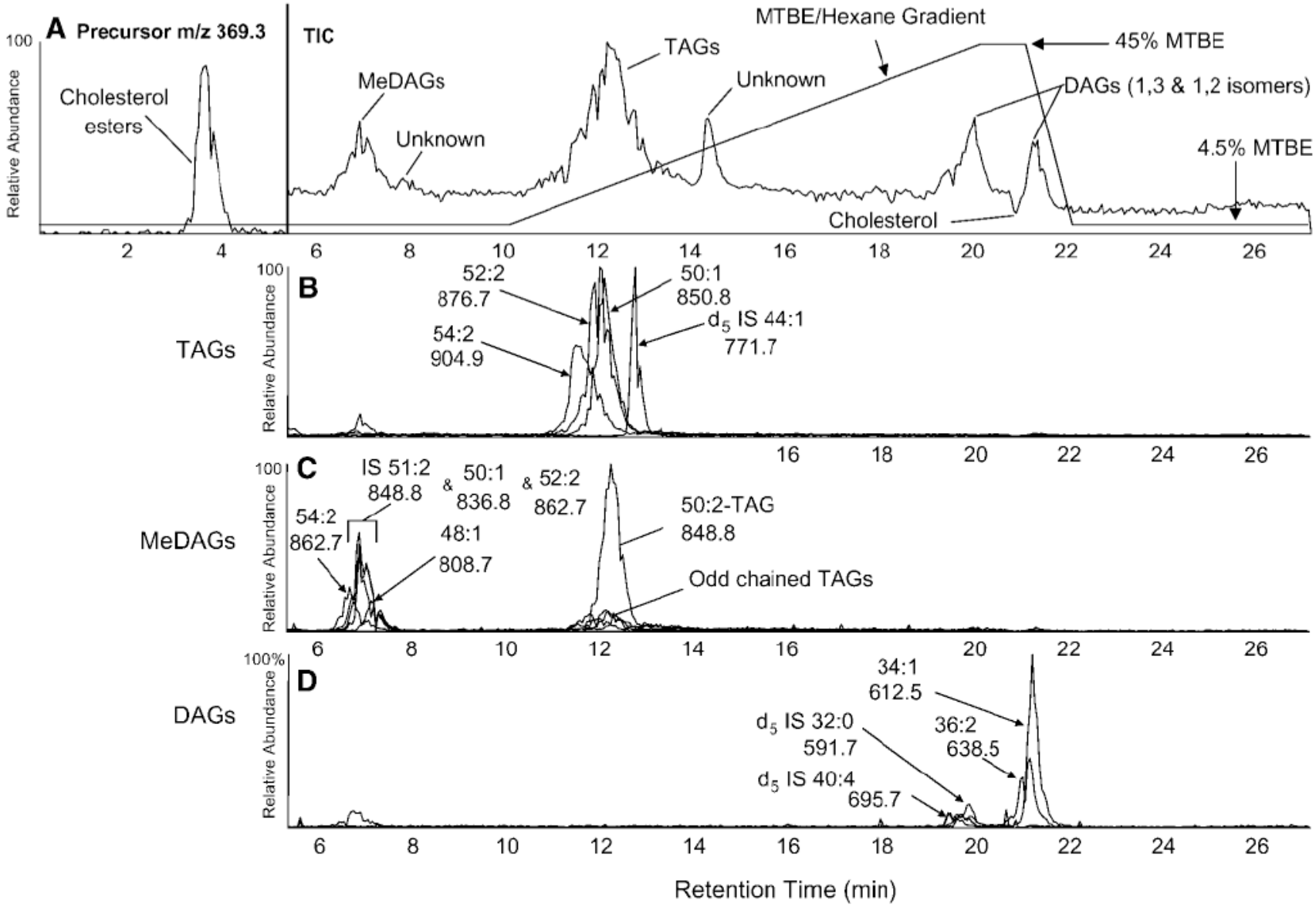
Stationary Phase – Silica gel ***Mobile Phase – MTBE***



What is Reversed-phase HPLC (RP-HPLC)?

Reversed-phase HPLC (RP-HPLC), separates analytes based on their hydrophobicity

Reverse(d) Phase Chromatography (RP-HPLC)

Interaction : Hydrophobic

Packing materials : Non-polar **ex. Silica-C18**
Silica-C8

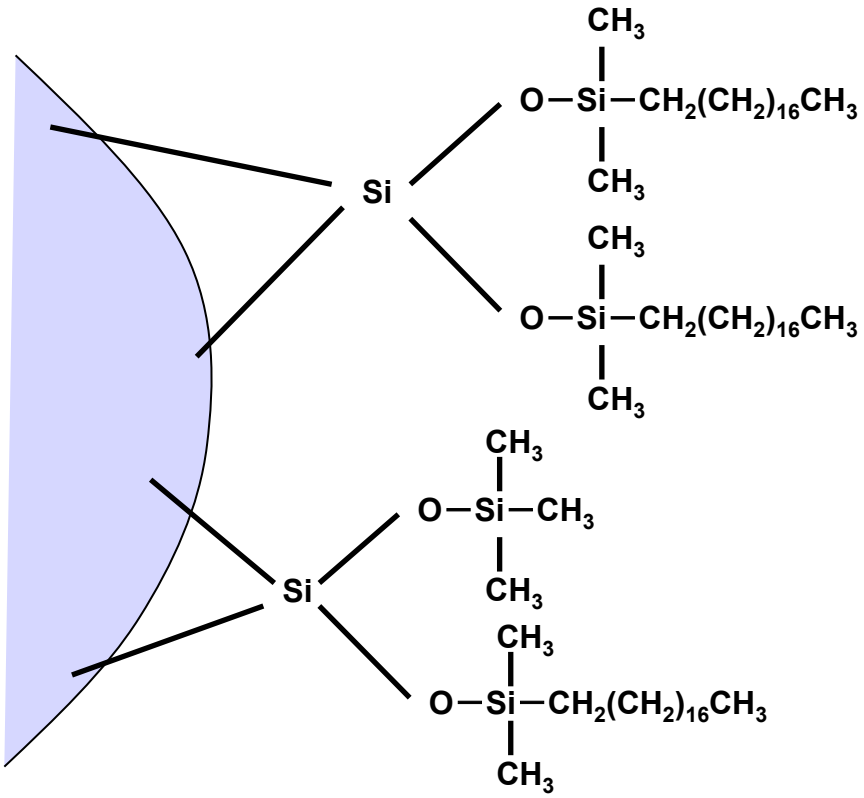
Mobile phase : Polar **ex. MeOH/H2O**
CH3CN/H2O
MeOH/Buffer sol.

Sample : Having different length of carbon chain

Reverse Phase Chromatography (RP-HPLC)

Silica-C18 Packing materials

Commonly used packing materials are hydrocarbons having 18 carbon atoms (called the Octadecyl radical) which are chemically bonded to silica gel (Silica-ODS).Since the surface of the Silica-ODS is covered with hydrocarbon, the polarity of the packing material itself is very low.



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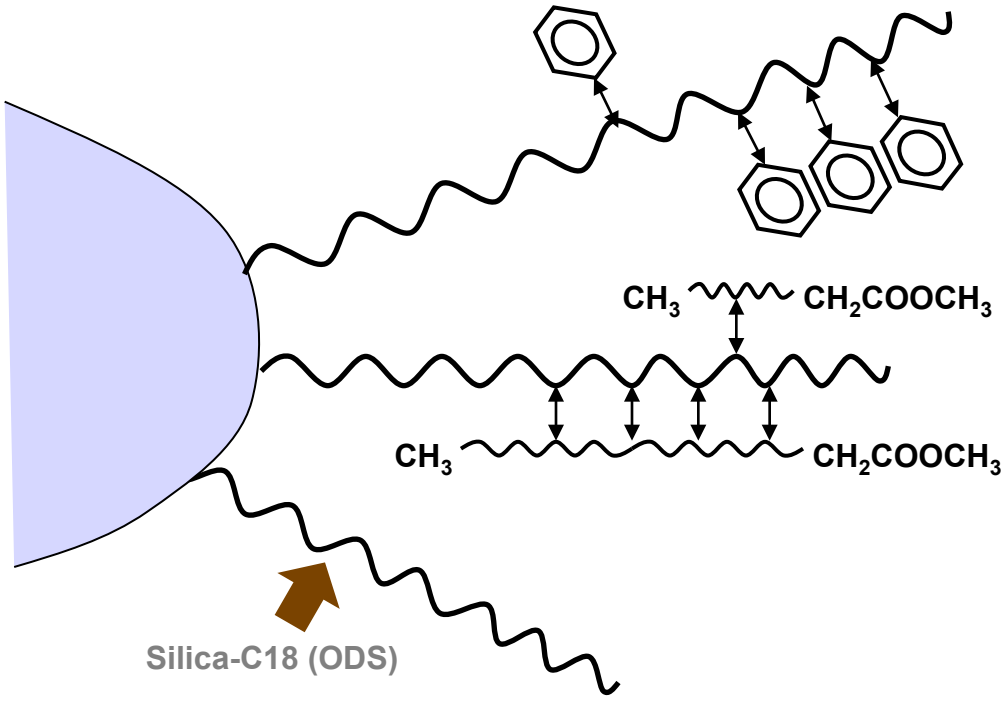
Reverse Phase Chromatography (RP-HPLC)

Hydrophobic Interaction



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Reverse Phase Chromatography (RP-HPLC)

Mobile phase solvents

Main solvent : MeOH - H2O
CH3CN - H2O

Sub solvent :EtOH
IPA
THF
DMF

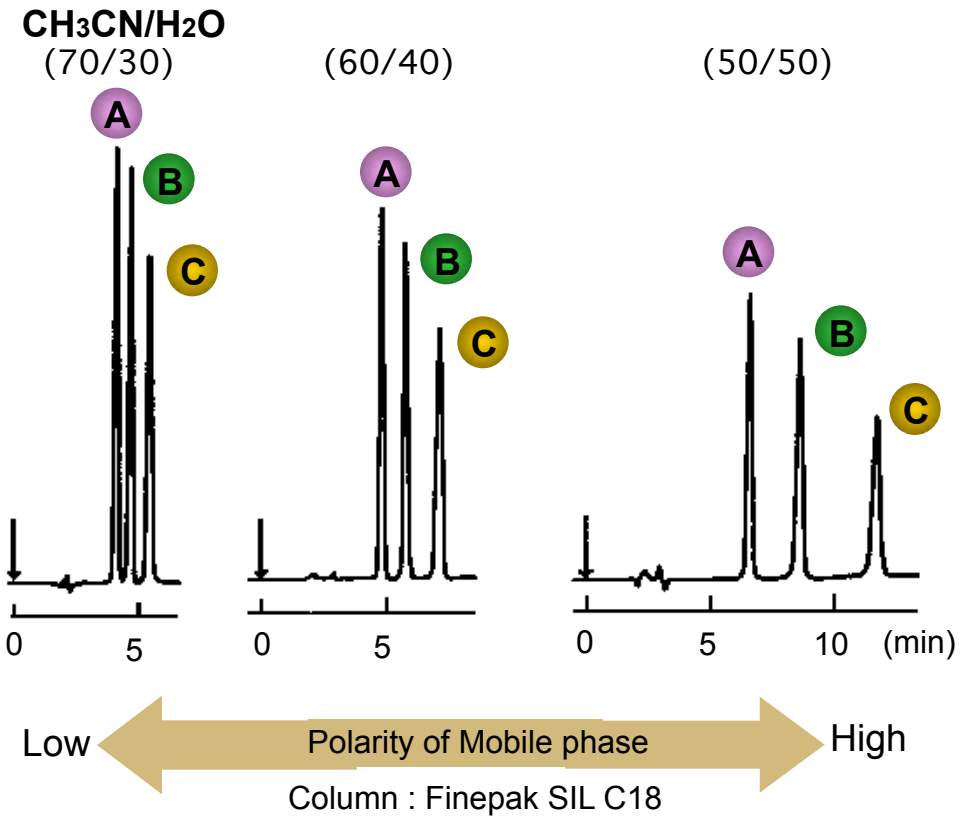
Additive : Acid
Salt
Ion-pairing agent

Reverse Phase Chromatography (RP-HPLC)

Retention behavior in reversed phase HPLC

Carbon chain length of sample

A < B < C



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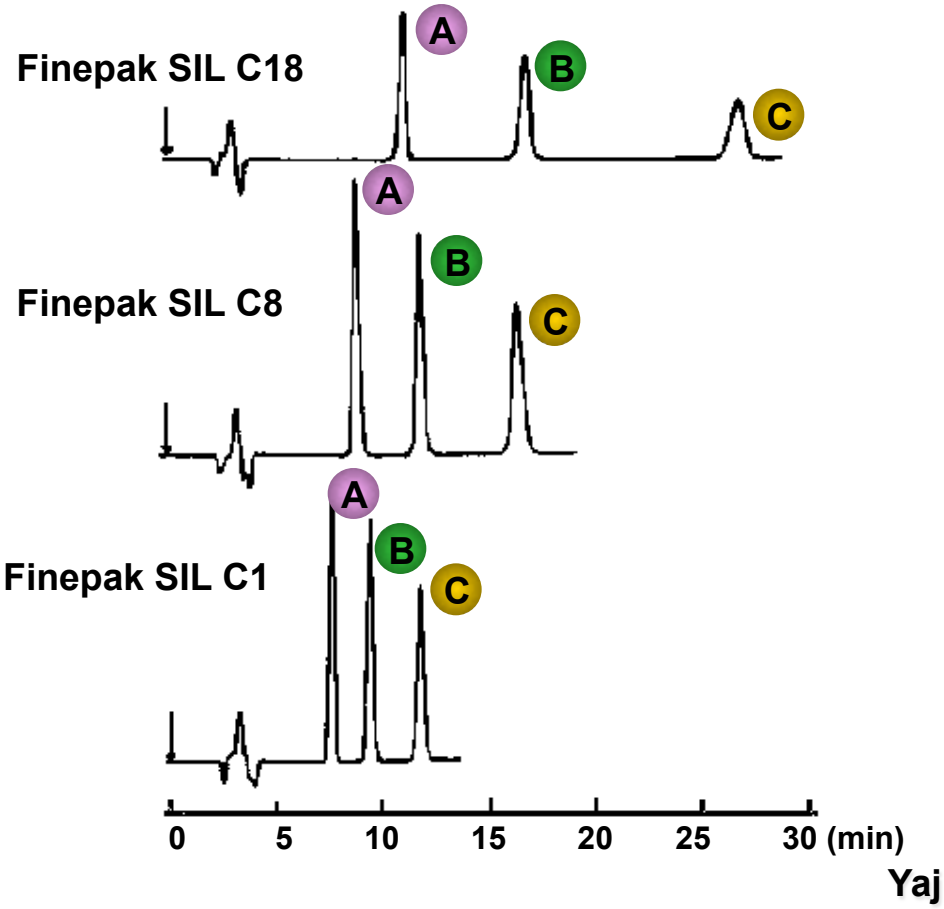
Reverse Phase Chromatography (RP-HPLC)

Length of packing materials carbon chains and retention time



Mobile phase : CH_3CN/H_2O(40/60)

A : p-Hydroxy ethyl benzoate

B : p-Hydroxy propyl benzoate

C : p-Hydroxy butyl benzoate

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Purification, Characterization, and *in Vitro* Mineralization Studies of a Novel Goose Eggshell Matrix Protein, Ansocalcin*

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Biomineralization is an important process in which hard tissues are generated through mineral deposition, often assisted by biomacromolecules. Eggshells, because of their rapid formation via mineralization, are chosen as a model for understanding the fundamentals of biomineralization. This report discusses purification and characterization of various proteins and peptides from goose eggshell matrix. A novel 15-kDa protein (ansocalcin) was extracted from the eggshell matrix, purified, and identified and its role in mineralization evaluated using *in vitro* crystal growth experiments. The complete amino acid sequence of ansocalcin showed high homology to ovocleidin-17, a chicken eggshell protein, and to C-type lectins from snake venom. The amino acid sequence of ansocalcin was characterized by the presence of acidic and basic amino acid multiplets. *In vitro* crystallization experiments showed that ansocalcin induced pits on the rhombohedral faces at lower concentrations (<50 μg/ml). At higher concentrations, the nucleation of calcite crystal aggregates was observed. Molecular weight determinations by size exclusion chromatography and sodium dodecyl sulfate -polyacrylamide gel electrophoresis showed reversible concentration-dependent aggregation of ansocalcin in solution. We propose that such aggregated structures may act as a template for the nucleation of calcite crystal aggregates. Similar aggregation of calcite crystals was also observed when crystallizations were performed in the presence of whole goose eggshell extract. These results show that ansocalcin plays a significant role in goose eggshell calcification.

the mineral phase is intimately associated with organic macromolecules, such as proteins/glycoproteins, polysaccharides, or proteoglycans (6). These biomacromolecules are highly acidic in nature and have been postulated to control nucleation, growth, crystal size, and shape of the mineral phases (7).

Mann (8) classified the biologically programmed composites into four types (types I, II, III, and IV), based on matrix intervention in nucleation and growth processes. Avian eggshells form type II biocomposites, which is the fastest forming hard acellular composite in nature. For example, in the case of chicken eggshell, about 5 g of the mineral phase is produced within 24 h (9). Their calcified layer consists of ~95% mineral and ~5% organic phase (10). The mineral phase acts as a mechanical support as well as allows the diffusion of gases, water, and ions and is, therefore, essential for survival of the embryo. The organic phase (proteins and proteoglycans) of the eggshell matrix is believed to be responsible for the nucleation and directed growth of the calcified layer. So far, several matrix proteins from chicken eggshells have been purified and characterized (11–17). These proteins are subdivided into three groups, namely non-collagenous bone proteins (osteopontin), eggshell-specific proteins (ovocleidins and ovocallyxins), and egg white proteins (ovalbumin, ovotransferrin, and lysozyme). Although their presence within the mineral layer has been demonstrated by immunohistochemistry (11, 12, and 17), the role of these proteins in calcite mineralization is not clearly understood. Hincke *et al.* (18) showed that egg white lysozyme and ovotransferrin influence the morphology of $CaCO_3$ crystals. Dermatan sulfate, chondroitin sulfate, and hyaluronic acid were also identified in the chicken eggshell matrix (19–22). Wu *et al.* (23) have shown that partially purified dermatan

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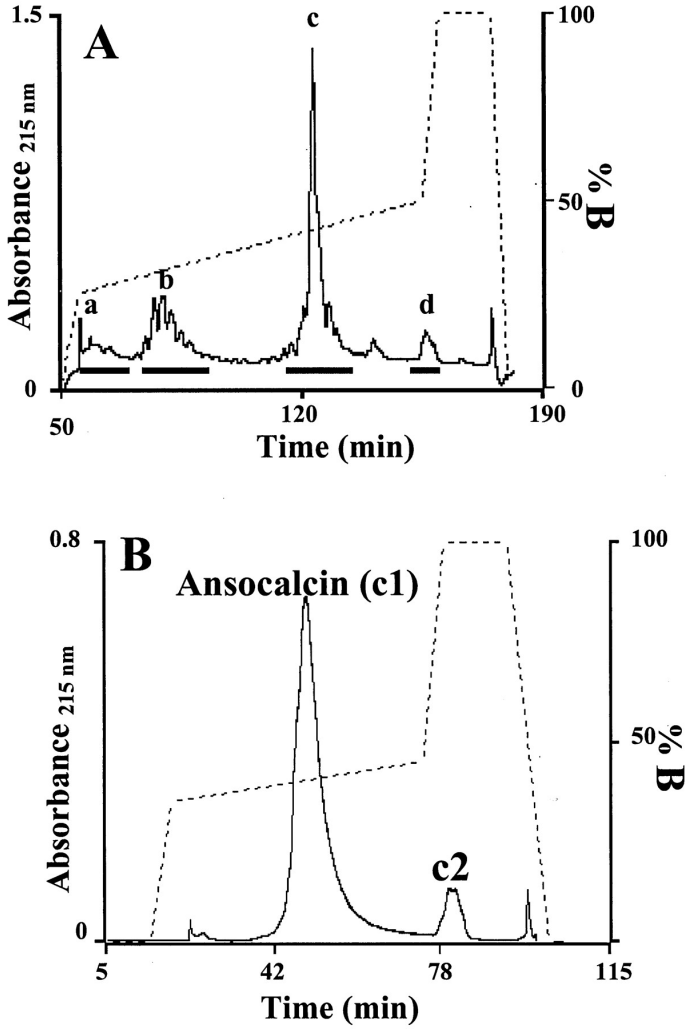
Purification of ansocalcin from goose eggshell extract.



Lakshminarayanan R et al. J. Biol. Chem. 2003;278:2928-2936

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Comparison of Reversed Phase and Normal Phase

	Normal phase	Reversed phase
Stationary phase	High polarity	Low polarity
Mobile phase	Low polarity	High polarity
Interaction	Adsorption	Hydrophobic
Elution order	Low to High (Polarity)	Short to Long (Length of Carbon chain)

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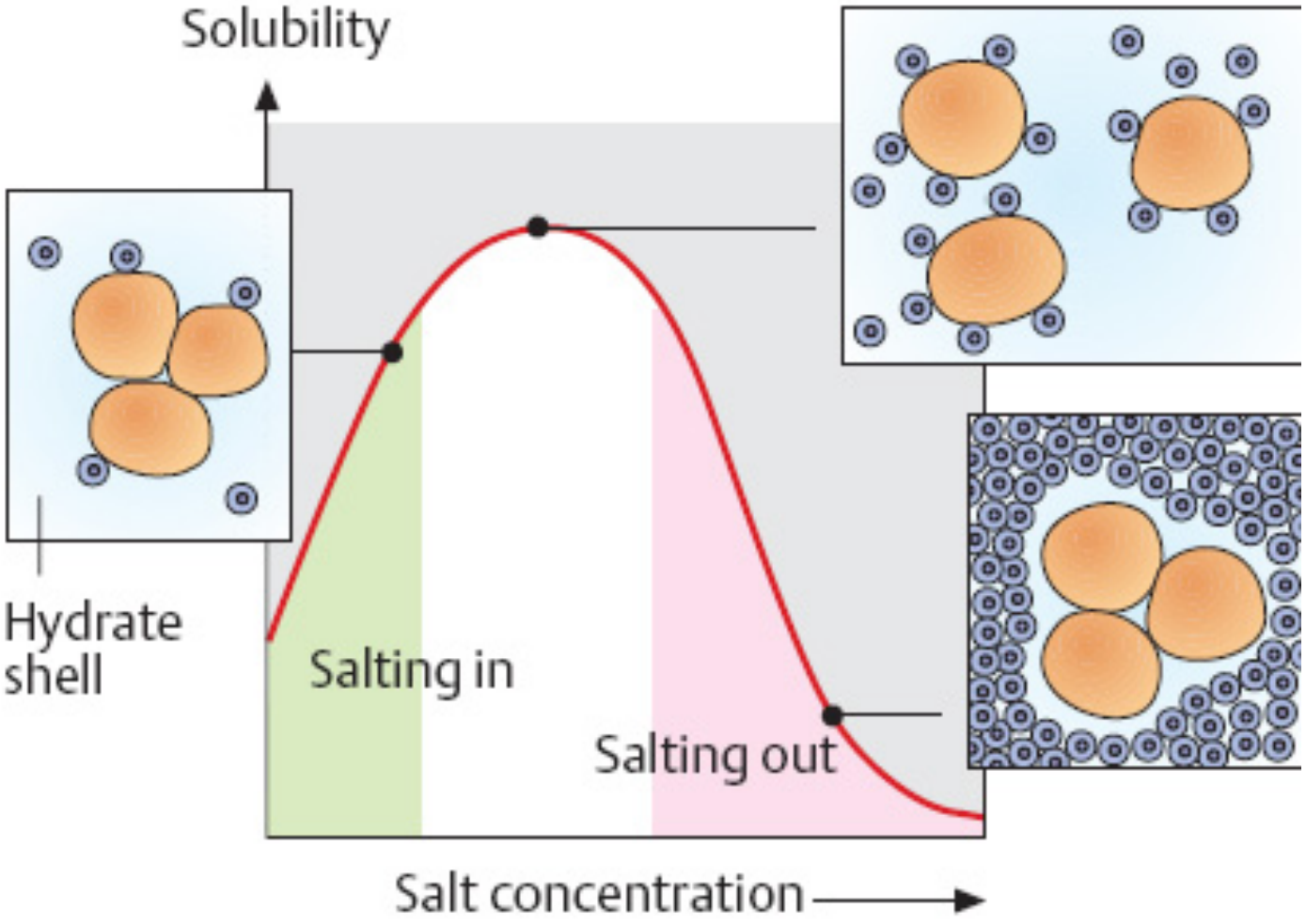
Salting out



At a specific salt concentration protein is hydrophobic enough not to precipitate but bind to a hydrophobic surface

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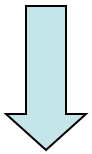
What is Hydrophobic Interaction Chromatography (HIC)?

Hydrophobic Interaction Chromatography (HIC) is a type of chromatograpic technique where molecules are separated on the basis of relative hydrophobicity.

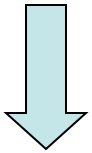
Steps of HIC

Load sample in high concentration of lyotropic salt



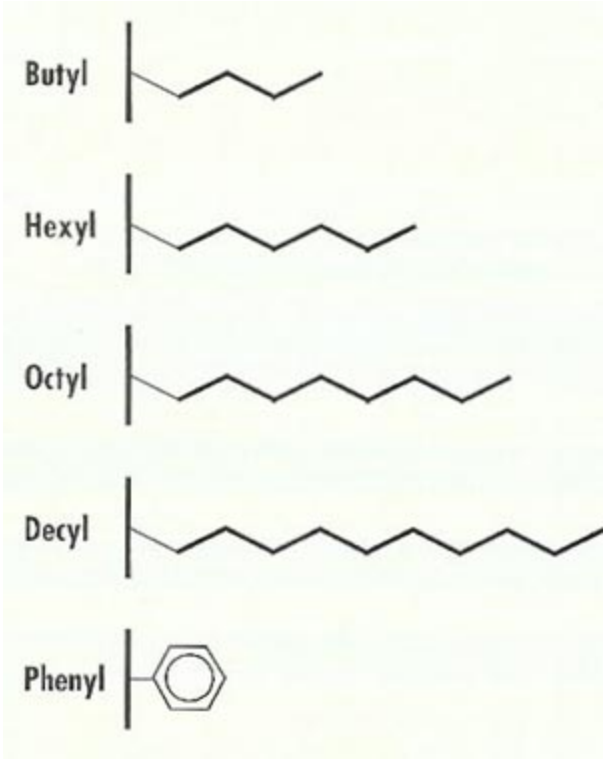
Protein molecules in the sample bind to the resin in order of their Hydrophobicity

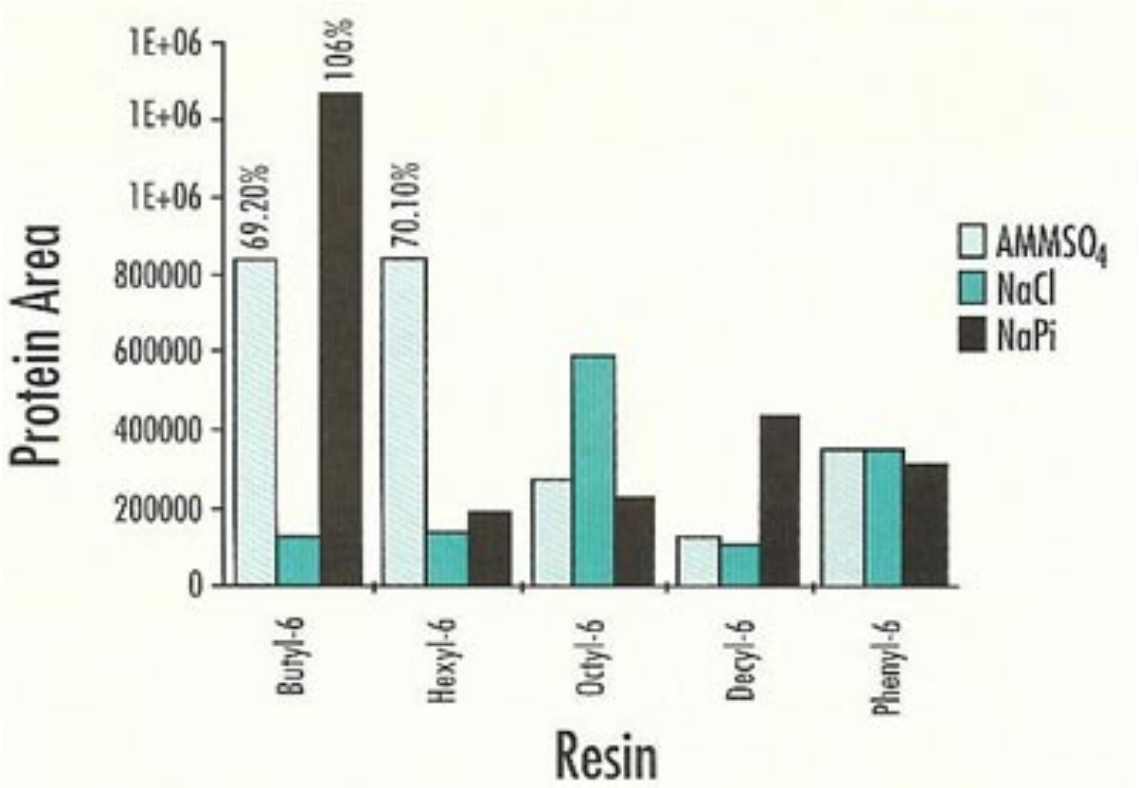
More Hydrophobic is the protein – lesser amount of a given Lyotropic salt



Elute in the buffer containing no Lyotropic salt

Binding of BSA to different resins in different lyotropic salts





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Purification and biochemical characterization of a specific β-glucosidase from the digestive fluid of larvae of the palm weevil, *Rhynchophorus palmarum*

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Abstract

A β-glucosidase was purified from the digestive fluid of the palm weevil *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) by chromatography on anion-exchange, gel filtration, and hydrophobic interaction columns. The preparation was shown to be homogeneous on polyacrylamide gels. β-glucosidase is a monomeric protein with a molecular weight of 58 kDa based on its mobility in SDS-PAGE and 60 kDa based on gel filtration. Maximal β-glucosidase activity occurred at 55°C and pH 5.0. The purified β-glucosidase was stable at 37°C and its pH stability was in the range of 5.0–6.0. The enzyme readily hydrolyzed *p*-nitrophenyl-β-D-glucoside, cellobiose, cellodextrins and required strictly β-gluco configuration for activity. It cleaved glucose-glucose beta-(1–4) linkages better than β-(1–2), β-(1–3) and β-(1–6) linkages. The catalytic efficiency (K_{cat}/K_M) values for *p*-nitrophenyl-β-D-glucoside and cellobiose were respectively 240.48 $mM^{-1}s^{-1}$ and 134.80 $mM^{-1}s^{-1}$. Beta-glucosidase was capable of catalysing transglucosylation reactions. The yield of glucosylation of 2-phenylethanol (20 %), catalysed by the beta-glucosidase in the presence of cellobiose as glucosyl donor, is lower than those reported previously with conventional sources of beta-glucosidases. In addition, the optimum pH is different for the hydrolysis (pH 5.0) and transglucosylation reactions (pH 6.6).

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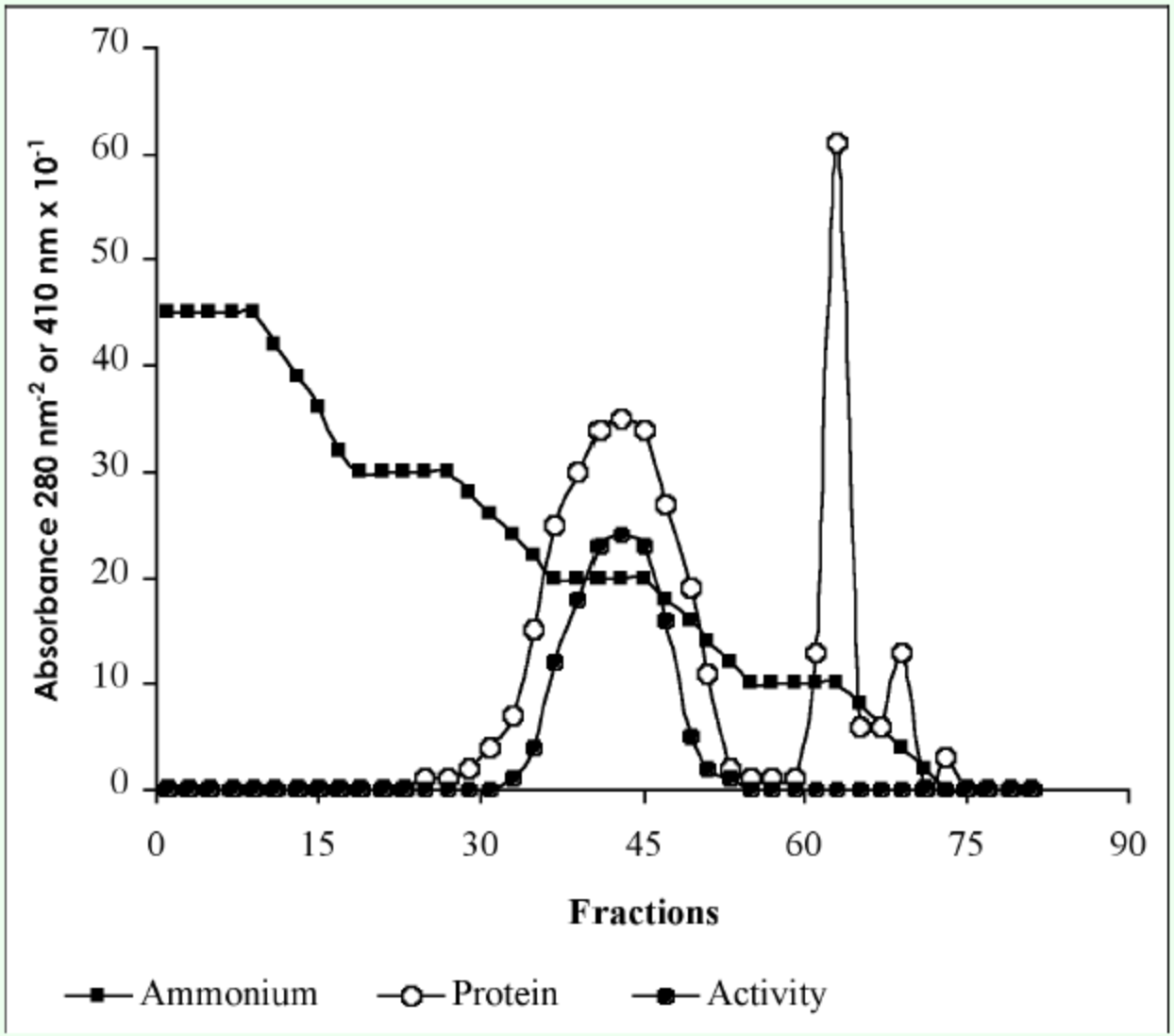

Figure 3. Hydrophobic interaction chromatography of β-glucosidase from the digestive juice of the palm weevil *Rynchophorus palmarum* larvae on Phenyl-Sepharose CL-6B.

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Chromatographic Modes of Protein Purification

Chromatographic Mode	Acronym	Separation Principle
Non-interactive modes of liquid chromatography		
Size-exclusion chromatography	**SEC**	Differences in molecular size
Interactive modes of liquid chromatography		
Ion-exchange chromatography	**IEC**	Electrostatic interactions
Normal-phase chromatography	**NPC**	Polar interactions
Reversed-phase chromtography	**RPC**	Dispersive interactions
Hydrophobic interaction chromatography	**HIC**	Dispersive interactions
Affinity chromatography	**AC**	Biospecific interaction
Immobilized Metal Affinity Chromatography	**IMAC**	Complex w/ an immobilized metal

(Christian G. Huber, Biopolymer Chromatography, Encylcopedia in analytical chemistry, 2000)

Society magazines

- Journal of Chromatography.
- Analytical Chemist


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Discussion