Přednáška 1

Imobilizované biologické systémy
Immobilized Biocatalysts

1. Introduction

- Working Party on Applied Biocatalysts within the European Federation of Biotechnology: “Immobilized biocatalysts are enzymes, cells, or organelles (or combinations of them) which are in a state that permits their reuse”

Historical Background:

( 1823 vinegar production, sludge, attachment to equipment )
50s – 60s : immobilization of enzymes
( 1916 Nelson – Griffin: invertase ads.on charcoal
1948 Sunmer: jack bean urease)
1950 – 1970: intensive investigations on immobilized enzymes and other proteins
  ( e.g.antigens -> affinity chromatography )
1969 – first industrial appt.of immobilized enzyme
Optical resolution of DL aminoacids with immobilized amino acylase
  ( Chibata et al. )
Since 1960 investigations on immobilized cells
Industrial applications of immobilized microbial cells:
1973    L – aspartic acid -Escherichia coli (aspartase )
1974    L - malic acid – Brevibacterium ammoniagenes
        ( fumarase)
1982    L – alanin – Pseudomonas dacunhae ( L-aspartate β - decarboxylase )
Introduction

- Biocatalysts dissolved in aqueous buffer solutions
- *soluble or native* enzymes, cells, cell parts, or organelles.
- *Immobilized, fixed, or insolubilized* enzymes, cells, etc., denote biocatalysts that are bound to a support.
- *carrier, support, or matrix*
- *cross-linking agent, bifunctional agent, or carrier activator*.
Membránové bílkoviny

Interakce membránových bílkovin s lipidovou dvojvrstvou: a) jeden transmembránový segment, b) více transmembránových segmentů, c) vazba periferní bílkoviny na bílkovinu integrální, d) vazba periferní bílkoviny pomocí elektrostatických interakcí, e) vazba pomocí hydrofobního koncového oligopeptidu, f) vazba pomocí kovalentně vázaného lipidu.
2.2. Methods of Enzyme Immobilization

1. modified into a water-insoluble form,

2. retained by an ultrafiltration membrane inside a reactor, or

3. bound to another macromolecule to restrict their mobility.
Imobilization Techniques

Figure 1. Classification of enzyme immobilization methods
A scheme of various ways of enzyme immobilization:

A - Binding of enzyme to an insoluble carrier,
B - Cross-linking,
C - Encapsulation of enzyme,
D - Entrapment of enzyme in gel structure.
Encapsulation of Enzyme
2.2.1. Carriers for Enzyme Immobilization

1. Large surface area and high permeability
2. Sufficient functional groups for enzyme attachment under nondenaturing conditions
3. Hydrophilic character
4. Water insolubility
5. Chemical and thermal stability
6. Mechanical strength
7. High rigidity and suitable particle form
8. Resistance to microbial attack
9. Regenerability
10. Toxicological safety
11. Low or justifiable price
**Table 2. Chemical classification of matrixes used for enzyme immobilization**

<table>
<thead>
<tr>
<th>Natural polymers</th>
<th>Minerals</th>
<th>Synthetic polymers</th>
<th>Synthetic materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharides</td>
<td>Attapulgite clays</td>
<td>Polystyrene</td>
<td>Nonporous glass</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Bentonite</td>
<td>Polyacrylates and poly-</td>
<td>Controlled pore glass</td>
</tr>
<tr>
<td>Starch</td>
<td>Kieselghur</td>
<td>methacrylates</td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>Pumice stone</td>
<td>Polyacrylamide</td>
<td>oxides</td>
</tr>
<tr>
<td>Agar and agarose</td>
<td></td>
<td>Hydroxyalkyl methacrylates</td>
<td>Metals</td>
</tr>
<tr>
<td>Alginate</td>
<td></td>
<td>Glycidyl methacrylates</td>
<td></td>
</tr>
<tr>
<td>Carrageenan</td>
<td></td>
<td>Maleic anhydride polymers</td>
<td></td>
</tr>
<tr>
<td>Chitin and chitosan</td>
<td></td>
<td>Vinyl and allyl polymers</td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td>Polyamides</td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon materials (activated carbon)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Polysaccharides** include cellulose, starch, and dextran.
- **Cellulose** and **Starch** are examples of natural polymers.
- **Minerals** include attapulgite clays, bentonite, kieselghur, and pumice stone.
- **Synthetic polymers** include polystyrene, polyacrylates and poly-methacrylates, polyacrylamide, hydroxyalkyl methacrylates, glycidyl methacrylates, maleic anhydride polymers, and vinyl and allyl polymers.
- **Synthetic materials** include nonporous glass, controlled pore glass, controlled pore metal, oxides, metals, and polyamides.
Agar

Agar, also called agar-agar, kanten, or gelose, is the oldest known gel-forming polysaccharide.

Discovered in the 17th century in Japan and consumed for 200 years, agar is extracted from certain marine red algae of the class Rhodophyceae mainly from *Gelidium* and *Gracilaria* species, growing essentially along the coasts of Morocco, Spain, Portugal, Chile, Japan and Korea.
Origin of seaweed extracts — general classification

a. Species of economic significance
b. Contains only component mentioned
c. Contains predominantly underlined component
Agar

Koch and Petri in 1882 - medium in which to grow bacteria no better solidifying agent in microbiological media has been found

microbiological, biotechnological, and public health laboratories, and an important colloid in other industries

permitted gelling, stabilizing, and thickening agent for food applications, authorized in all countries without limitations of daily intake (confectionery, bakery, pastry, beverage, sauces, wines, spreads, spices and condiments, meats and fishes, dairy, jams, etc.)

Apart from its ability to gelify aqueous solutions and produce gel without the support of other agents, agar can also be used as a safe source of dietary fiber since it is not digestible by the human body.
Flow sheet of traditional agar extraction

Extraction
Purification
Dehydratation
Structure of agar

**Agarose**

1,4-linked 3,6-anhydro-\(\alpha\)-l-galactose alternating with 1,3-linked \(b\)-d-galactose

**Agarpectin**

repeating unit as agarose, some of the 3,6-anhydro-l-galactose residues can be replaced with l-galactose sulfate residues and the d-galactose residues are partially replaced with the pyruvic acid acetal 4,6-\(O\)-(1-carboxyethylidene)-d-galactose
Agar
Agar

Agar cell walls of red seaweeds: D-galactose 3,6-anhydro-L-galactose few sulfate groups
Agar

Agar consists of a mixture of agarose and agaropectin. Agarose is a linear polymer, of molecular weight about 120,000, based on the -(1®3)-b-D-galactopyranose-(1®4)-3,6-anhydro-a-L-galactopyranose unit; the major differences from carrageenans being the presence of L-3,6-anhydro-a-galactopyranose rather than D-3,6-anhydro-a-galactopyranose units and the lack of sulfate groups. Agaropectin is a heterogeneous mixture of smaller molecules that occur in lesser amounts. Their structures are similar but slightly branched and sulfated, and they may have methyl and pyruvic acid ketal substituents. They gel poorly and may be simply removed from the excellent gelling agarose molecules by using their charge. The quality of agar is improved by alkaline treatment that converts of any L-galactose-6-sulfate to 3,6-anhydro-L-galactose.
Agarosa

Processing to remove $\text{SO}_3^- \text{ NaBH}_4^-/-\text{OH}$

- Macroporous, hydrophilic
- Commercial availability
- Chemically stable
- Low non-specific binding
- Resistant to MO
Agarosa

Matrice

Základní vlastnosti matrice:

1. Nerozpustnost v použitých rozpouštědlech a pufrech
2. Snadná derivatizace a následná vazba ligandu nebo raménka
3. Mechanicky, termicky a chemicky stabilní s dobrými průtokovými vlastnostmi (povrch větší než 10 m²/g, vysoká poresita, malé póry)
4. Hydrofilní charakter
5. Rezistentní vůči mikroorganismům
6. Schopnost regenerace

<table>
<thead>
<tr>
<th>název</th>
<th>adsorpční kapacita</th>
<th>% síry</th>
</tr>
</thead>
<tbody>
<tr>
<td>komerční agarosa 6%</td>
<td>0.080</td>
<td>0.118</td>
</tr>
<tr>
<td>ECD- agarosa</td>
<td>0.008</td>
<td>0.021</td>
</tr>
<tr>
<td>komerční agar 6% (částice)</td>
<td>0.240</td>
<td>0.371</td>
</tr>
<tr>
<td>ECD- agar 6% (částice)</td>
<td>0.060</td>
<td>0.049</td>
</tr>
<tr>
<td>redukovaný ECD agar 6% částice</td>
<td>0.004</td>
<td>0.012</td>
</tr>
</tbody>
</table>
Agarose

- Agarose molecules have molecular weights about 120,000, The gel network of agarose contains double helices formed from left-handed threefold helices. These double helices are stabilized by the presence of water molecules bound inside the double helical cavity [508]. Exterior hydroxyl groups allow aggregation of up to 10,000 of these helices to form suprafibers.
Agarosa

Zlepšení mechanických vlastností

Prokřížení např. epichlorhydrinem
Gelling mechanism

Gel formation mechanism in aqueous agar solutions

Hysteresis of 1.5% agar gels

Three equatorial hydrogen atoms of the 3,6-anhydro- α-l-galactose residue are responsible for constraining the molecule so as to form a helix with a threefold screw axis
Quick Soluble Agar

Comparison of production processes of traditional agar and QSA

Patent manufacturing process without any chemical or genetic modifications
Alginate

family of linear 1,4-linked $\alpha$-L gulurono-$\beta$-\(\text{D}\)-mannuronans of widely varying composition and sequential structures

Commercial preparations are usually designated as alginates and include alginic acid, its salts, and derivatives
Origin of seaweed extracts — general classification

- a. Species of economic significance
- b. Contains only component mentioned
- c. Contains predominantly underlined component
Flow sheet for the production of sodium alginate
\[ 4C_1: \text{eq-eq} \]

\[ 1C_4: \text{ax-ax} \]

\[ 1C_4: \text{ax-eq} \quad 4C_1 \]

\[ (1,4)-\beta-D-Mannuronic \text{ acid}-(1,4)-\beta-D-mannuronic \text{ acid}- (1,\]

\[ (1,4)-\alpha-L-Glucuronic \text{ acid}-(1,4)-\alpha-L-glucuronic \text{ acid}- (1,\]

\[ (1,4)-\alpha-L-Glucuronic \text{ acid}-(1,4)-\beta-D-mannuronic \text{ acid}- (1,\]
Alginates are linear unbranched polymers containing β-(1-4)-linked D-mannuronic acid (M) and α-(1-4)-linked L-guluronic acid (G) residues. Although these residues are epimers (D-mannuronic acid residues being enzymatically converted to L-guluronic after polymerization and only differ at C5, they possess very different conformations; D-mannuronic acid being 4C1 with diequatorial links between them and L-guluronic acid being 1C4 with diaxial links between them. Bacterial alginites are additionally O-acetylated on the 2 and/or 3 positions of the D-mannuronic acid residues. The bacterial O-acetylase may be used to O-acetylate the algal alginites, so increasing their water binding.
Structure of Alginate

structural elements:
homopolysaccharides $\alpha$-1,4-\text{L}-guluronan and $\beta$-1,4-\text{D} mannuronan
heteropolysaccharide consisting of alternating 1,4-linked $\alpha$-\text{L}-guluronic (G) and $\beta$-\text{D}-mannuronic acid (M) residues

$$- M - G - M - (M - M)n - M - G - (M - G)q - M - G - (G - G)p - G - M - G -$$
Alginate

GGGGGGGGGG

MMMMMMMMM

GMGMGMGMGM
in Alginate

Fig. 1. Apparatus for entrapment in calcium alginate.
Alginate

Fig. 8.15. Chain association by complex formation with calcium ions: the oxygen atoms that form chelate bonds are designated by O. (Reproduced from Ref. 41 with permission.)
- ve vodě rozpustný alginát sodný
- zjemňovadlo, emulsifikátor, jako film, želírující látka
- netoxický, laciný
- biochemicky inertní, mechanicky stabilní
Alginate

- Alginate's solubility and water-holding capacity depend on pH (precipitating below about pH 3.5), molecular weight (lower molecular weight calcium alginate chains with less than 500 residues showing increasing water binding with increasing size), ionic strength (low ionic strength increasing the extended nature of the chains) and the nature of the ions present. Generally alginates show high water absorption and may be used as low viscosity emulsifiers and shear-thinning thickeners. They can be used to stabilize phase separation in low fat fat-substitutes e.g. as alginate/caseinate blends in starch three-phase systems. Alginate is used in a wide variety of foodstuff such as pet food chunks, onion rings, stuffed olives, low fat spreads, sauces and pie fillings. Propylene glycol alginates have widespread use as acid-stable stabilizers for uses such as preserving the head on beers.
Alginate

- The primary function of the alginates are as thermally stable cold setting gelling agents in the presence of calcium ions; gelling at far lower concentrations than gelatin. Such gels can be heat treated without melting, although they may eventually degrade. Gelling depends on the ion binding (Mg2+ << Ca2+ < Sr2+ < Ba2+) with the control of the dication addition being important for the production of homogeneous gels (e.g. by ionic diffusion or controlled acidification of CaCO3). High G content produces strong brittle gels with good heat stability (except if present in low molecular weight molecules) but prone to water weepage (syneresis) on freeze-thaw, whereas high M content produces weaker more-elastic gels with good freeze-thaw behavior and high MGMG content zips with Ca2+ ions to reduces shear. However, at low or very high Ca2+ concentrations high M alginates produce the stronger gels. So long as the average chain lengths are not particularly short, the gelling properties correlate with average G block length (optimum block size ~12; see also the similarity to pectin gelling) and not necessarily with the M/G ratio which may be primarily due to alternating MGMG chains. The future prospects are excellent as recombinant epimerases with different specificities may be used to produce novel designer alginates.
Sodium alginate is the important material to produce seaweed products such as man-made grape and manmade cherry.

b. iced food

Sodium alginate is as a stabilizer of ice cream because of its dense organization is very equal and the slow solution speed. It is the main material of the dainty cold powder and jelly.
Application

- c.cake food
  As a stabilizer of cake (such as cookies, bread, fine dried noodle, chocolates etc) and a polish of bread. Sodium alginate can keep the cookies sweet-smelling and avoid falling to pieces, and make the noodle smooth, reduce breaking ratio of noodle and fragmentation of bread.
Application

- Sodium alginate is also the good thickener of jam, chili sauce, jelly, tomato ketchup, fishpaste, pudding and sala flavoring.
- Sodium alginate is as the stabilizer of the beer and clarifier of the wine.
- Freezing and keeping fresh
  When food (fruit, fish etc) is covered with the film of sodium alginate and kept isolated from air, the film will stop bacteria from invading, constraint water evaporation of food itself and prolonged time of preservation.
Origin and main sugar moieties of polysaccharides

Pectin cell walls and middle lamella of higher land plants: D-galacturonic acid D-galacturonic acid methylester

-d-Galactopyranosyluronic acid in $^4C_1$ conformation (above); fragment of galacturonan chain, 40 % methylate
Pectin

Schematic representation of pectin backbone, showing the “hairy” regions (rhamnogalacturonan and side-chains) and the “smooth” regions (linear galacturonan)
Alginate cells walls of brown seaweeds exopolysaccharides of *Azetobacter vinelandii*: mannuronic acid L-guluronic acid and their acetyl derivatives

Carrageenan cell walls of red seaweeds: D-galactose 3,6-anhydro-D-galactose both sugars sulfated to higher or lower degree
Carageenan

- 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units
Carrageenan

- Carrageenan (E407) is a collective term for polysaccharides prepared by alkaline extraction (and modification) from red seaweed (Rhodophycae), mostly of genus Chondrus, Eucheuma, Gigartina and Iridaea. Different seaweeds produce different carrageenans.
κ-carrageenan (kappa-carrageenan) -(1-3)-β-D-galactopyranose-4-sulfate-(1-4)-3,6-anhydro-α-D-galactopyranose-(1-3)
ι-carrageenan (iota-carrageenan)-(1-3)-β-D-galactopyranose-4-sulfate-(1-4)-
3,6-anhydro-α-D-galactopyranose-2-sulfate-(1-3)-
\[ \lambda \text{-carrageenan} \text{ (lambda-carrageenan)} - (1-3) - \beta \text{-D-galactopyranose-2-sulfate-} \\
(1-4) - \alpha \text{-D-galactopyranose-2,6-disulfate-(1-3)} \]
Gelation of carrageenans by formation of double helices and aggregation of helices
Caragenan

Dried red seaweeds
Alkaline solution (ca. 130°C)
Crude extract
Sieving Filtration (activated carbon)
Purified extract 1.5%
Vacuum concentration
Concentrate 3%
Alcohol precipitation Alcohol washing Vacuum drying
Drum drying
Crude carageenan
Grinding, standardization
Standardized commercial products
κ-Carrageenan stabilizes milk k-casein products due to its charge interaction with the casein micelles (~200 nm diameter); their incorporation into the network preventing whey separation. Such complexes are soluble when both have same charge and are held together by counterions or oppositely charged patches. Carrageenan is also used as a binder in cooked meats, to firm sausages and as a thickener in toothpaste and puddings.
Cellulose

- β-(1-4)-D-glucopyranose units in $4C_1$ conformation
Cellulose
Carboxymethylcellulose
Differential mass distribution curves of various celluloses

- a) Cotton;
- b) Cotton
- c) China grass (ramie)
- d) Flax
- e) Balsam
- f) White fir
Degree of polymerization of celluloses of different origin

<table>
<thead>
<tr>
<th>Type of cellulose</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton, raw</td>
<td>7 000</td>
</tr>
<tr>
<td>Cotton, raw (according to Russian work)</td>
<td>14 000</td>
</tr>
<tr>
<td>Cotton, purified</td>
<td>1 500 – 300</td>
</tr>
<tr>
<td>Cotton linters</td>
<td>6 500</td>
</tr>
<tr>
<td>Flax</td>
<td>8 000</td>
</tr>
<tr>
<td>Ramie</td>
<td>6 500(a)</td>
</tr>
<tr>
<td>Cellulose (isolated from wood fibers)</td>
<td>1 100 – 800</td>
</tr>
<tr>
<td>Spruce, pulped</td>
<td>3 300</td>
</tr>
<tr>
<td>Beech, pulped</td>
<td>3 050</td>
</tr>
<tr>
<td>Aspen</td>
<td>2 500</td>
</tr>
<tr>
<td>Fir</td>
<td>2 500</td>
</tr>
<tr>
<td>Bacterial cellulose</td>
<td>2 700</td>
</tr>
<tr>
<td>Acetobacter cellulose</td>
<td>600</td>
</tr>
</tbody>
</table>
Electron micrograph of the fibrillar nature of cellulose fibers
The architecture of elementary fibrils and microfibrils of native celluloses
Fringe fibrillar model of fiber structure
Stability of some polysaccharides at various pH. Residual viscosity after 10 min incubation at 90 °C

a) Carboxymethylcellulose
b) Locust bean gum;
c) Agar;
d) Carrageenan;
e) Pectate;
f) Pectin
Chitin/ chitosan

\[ \beta-(1\rightarrow4) 2\text{ acetamido}-2\text{-deoxy } \beta-D\text{-D glucopyranosid} \]

\[ \xrightarrow{\text{HCl}} \]

2 amino – 2 deoxy glucosa
Chitin
Dextran

Figure 2.2. Schematic representation of agarose gel network (right), in comparison with a network such as Sephadex that is formed from random chains at similar polymers concentration. Note that the aggregates in agarose gels may actually contain $10^2$ to $10^4$ helices rather than the smaller numbers shown here. Reprinted with permission from Arnott, et al (1974), J. Molec. Biol. 90, 269. Copyright by Academic Press Inc. (London) Ltd.

From: W.H. Scouten
Affinity Chromatography

Dextran "Sephadex"

1,6-α-D-glucopyranose