Introduction to Starch

Starch

- Carbon and energy storage material for plants
- A major component of food and feed, providing source of energy and carbon
- Used for texturizing and to provide specific functionalities in processed foods
- Starting material for ethanol production
- Broad applications in the paper and textile industries

Industrial Starch Sources

Corn starch
- Common corn
- Waxy maize (amylose – 0%)
- High-amylose (amylose 55 and 70%)
- Other mutants

Root/Tuber starch
- Potato
- Cassava (Tapioca)

Other Cereals
- Wheat
- Rice
- Barley
Partial list of documented starch mutants of maize

<table>
<thead>
<tr>
<th>Single mutants</th>
<th>Double mutants</th>
<th>Triple/quadruple mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waxy (wx)</td>
<td>ae wx</td>
<td>ae du1 su1</td>
</tr>
<tr>
<td>Amylose-extender (ae)</td>
<td>ae su1</td>
<td>ae du1 su2</td>
</tr>
<tr>
<td>sbe1</td>
<td>ae su2</td>
<td>ae su1 su2</td>
</tr>
<tr>
<td>sbe2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugary-1 (sur1)</td>
<td>as du1</td>
<td>as su1 wx</td>
</tr>
<tr>
<td>Zpr1</td>
<td>du1 su1</td>
<td>as su2 wx</td>
</tr>
<tr>
<td>Sugary-2 (sur2)</td>
<td>du1 su2</td>
<td>as su1 ws</td>
</tr>
<tr>
<td>Dull-1 (dul1)</td>
<td>sur1 wx</td>
<td>du1 su1 su2</td>
</tr>
<tr>
<td>Brittle-1 (bt1)</td>
<td>sur2 wx</td>
<td>du1 su1 su2</td>
</tr>
<tr>
<td>Brittle-2 (bt2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shrunken-1 (sh1)</td>
<td>sur1 su2</td>
<td>su1 su2 wx</td>
</tr>
<tr>
<td>Shrunken-2 (sh2)</td>
<td></td>
<td>as du1 su1 su2</td>
</tr>
</tbody>
</table>

World starch production and utilizations

<table>
<thead>
<tr>
<th>Starch Production</th>
<th>Refinery Products</th>
<th>Starch Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn 83%</td>
<td>Dextrose and maltose</td>
<td>Paper (ca. 60%)</td>
</tr>
<tr>
<td>Potato 6%</td>
<td>High fructose corn syrup</td>
<td>Food (ca. 20%)</td>
</tr>
<tr>
<td>Wheat 6%</td>
<td>Maltodextrin</td>
<td>Textile (ca. 20%)</td>
</tr>
<tr>
<td>Tapioca 4%</td>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Others 1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

End-use demand for starch in major markets

<table>
<thead>
<tr>
<th></th>
<th>US</th>
<th>EU</th>
<th>Japan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper and paperboard</td>
<td>10</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>High-fructose sweetener</td>
<td>31</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td>Other sweeteners</td>
<td>12</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Fuel ethanol</td>
<td>42</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Food</td>
<td>3</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>Other industrial</td>
<td>2</td>
<td>26</td>
<td>10</td>
</tr>
</tbody>
</table>

*Corn Annual 2001, Corn Refiners association
Introduction to Starch

Starch granules

Normal light

Polarized light

Birefringence indicates that the granules are semi-crystalline
Introduction to Starch

Starch granules

Two populations of wheat starch: large size A granules, and small size B granules

Starch granule size distributions
Overview of Starch Granule

Amylose and Amylopectin

Amylose
- A linear molecule comprising of 1,4 linked alpha-D-glucopyranosyl units. There is a small degree of branching by 1,6 alpha linkages.
- The smaller of the two polysaccharides making up starch.

Amylopectin
- A highly branched molecule comprising both 1,4 linked and 1,6 linked alpha-D-glucopyranosyl units.
- Branches are non-randomly distributed in clusters.
- The larger of the two polysaccharides making up starch.
**Amylose and Amylopectin**

**Reducing end**

**Open ring**

**Aldehyde oxidized to carboxylic acid**

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**Amylose and Amylopectin**

Different type of chains for amylopectin

- Three chain types A, B and C
- A chains are not branched
- B chains are branched
- C chain has the reducing end R
- One reducing end per amylopectin molecule

Wang et al., J. Exp. Botany (1998) 49, 481

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**Amylose and Amylopectin**

Structural parameters of amylopectin clusters

Amylose and Amylopectin

Starch-lipid complex

Polar (hydrophilic) head outside
Nonpolar (hydrophobic) tail inside


Starch Crystallinity

Crystalline forms

- A: Cereal starch
- B: Tuber starch, high amylose starch
- V: amylose crystallized with lipids, iodine, etc
- Vh: hydrated V form


Starch Crystallinity

Crystalline A structure

Crystalline A structure for starch. An (a,b) plane projection of the unit showing helix packing, water molecules, and hydrogen bonding

Starch Crystallinity

Crystalline B structure

Crystalline B structure for starch. An (a,b) plane projection of the unit showing nearby helices and the center channel of organized water molecules.

Imberty et al. Biopolymers (1988) 27,1205

Amylose and Amylopectin

Estimation of Crystallinity $\alpha$

$\alpha = \frac{435}{1460} = 30\%$

Diagram from Dr. Rangaswami Chandrasekaran

Starch Biosynthesis and Genetic Starch Modifications of Maize
An introduction from maize kernel to amylopectin clusters
- Functional behaviors of enzymes synthesizing starch
- Mutant starches of maize, single mutants
- Mutant starches of maize, multiple mutants
- Mutant starches of maize, patents associated with foods
- Genetic starch modifications, challenges
- Genetic starch modifications, our approaches

Starch formation in plastids
- Starch granules are classified as transitory or reserve
- Transitory starch granules accumulate for only a short period of time before they are degraded, e.g.
  - Starch forms in leaf chloroplasts during the day
  - And hydrolyzed and transported to other plant parts at night as simple sugar
- Reserve starch, an energy storage for germination, a major component of food and feed, and an industrial starting material, is formed in amyloplasts
- We will be discussing starch biosynthesis in maize endosperm
From Maize Kernel to Amylopectin Clusters

Comparison of kernels and starch granules of two wx mutants

Genetic modifications provide diverse kernel phenotypes, starch granule sizes, internal granular structures, and starch molecular structures

Let's look at the big picture before “zooming in”......

Gallant et al, 1997, Carbohydrate Polymer, 32:177-191

Amylopectin branches are non-randomly clustered

Thompson, 2000, Carbohydrate Polymers, 43: 223-239
Structural parameters of amylopectin clusters

Keywords: amylopectin, clusters, branches, A chains, B chains, branching pattern


Chain length profiles are used to calculate AP parameters

From Maize Kernel to Amylopectin Clusters

What can genetic starch modifications do for us?

- Improve the starch yield of major crops
- Acquire starches with desirable functionalities and high value
  - Suitable starting materials for chemical and enzymatic modifications
  - Modified digestibility or degradability as food, feed, and industrial raw materials
  - Retarded or enhanced retrogradation after cooking, leading to extended shelf life or unique functionalities
  - Unique granular and nano structures for high-end uses, e.g. as carriers for controlled release
Successful genetic starch modification is based on the knowledge of starch biosynthesis

- Where are starch granules synthesized?
- What enzymes are involved in starch biosynthesis?
- What are the functional behaviors of these enzymes?
- Can we tailor starch structure for desirable functions?

Enzymes Synthesizing Starch

Starch biosynthesis in a cell of maize endosperm

Enzymes Synthesizing Starch

ADP-glucose pyrophosphorylase (AGPase)
Starch synthase (SS)
Isoforms identified: GBSSI, SSI, SSIIa, SSIIb, & SSIII

Soluble starch synthase (SSS) responsible for amylopectin synthesis
Granule-bond starch synthase (GBSS) responsible for amylose synthesis

Starch branching enzyme (SBE)
Isoforms identified: SBEI, SBEIIa, & SBEIIb

Starch debranching enzyme (DBE)
Isoforms identified: Isoamylase-like (SU1) & Pullulanase-like (ZPU1)
Enzymes Synthesizing Starch

Specific substrates may detect enzyme specificity

1. Plant tissue
2. Protein extraction
3. Native PAGE
4. Electroblotting
5. Incubation
6. Iodine staining
7. Photo recording

All SBEs may branch both amylose & amylopectin
SBEI preferentially branches amylose
SBEIIa & SBEIIb preferentially branches amylopectin

Starch branching enzymes
change gel from blue to purple or yellow

Potato starch
Waxy maize starch
Liver glycogen

Starch debranching enzymes
change gel from purple to blue

Shown here is a pullulanase-like DBE (ZPU1)
An isoamylase-like DBE (SU1) is not shown
Enzymes Synthesizing Starch

Starch synthases
change gel from yellow to dark blue

Potato starch Waxy maize starch Liver glycogen

At least 5 bands are shown here, with different activities
We will assign these bands to known SS isoforms

Mutant Starches from Maize

A list of documented starch mutants of maize

Single mutants                  double mutants                  Triple/quadruple mutants
Waxy (wx)                       ae wx                           as du1 su1
Amylose-extender (ae)           sbe1 wx                           ae du1 su1
sbe2a                              ae su1                          as du1 su2
Sugary-1 (su1)                   ae su2                              ae du1 wx
Zpu1                                 ae du1                             as su1 su2
Sugary-2 (su2)                   du1 su1                           sbe1 ae wx
Dull-1 (du1)                      du1 wx                           du1 su1 su2
Brittle-1 (bt1)                   su1 wx                             du1 su1 wx
Brittle-2 (bt2)                   su2 wx                           du1 su2 wx
Shrunken-1 (sh1)                  su1 su2                          su1 su2 wx
Shrunken-2 (sh2)                  su1 su2                          as du1 su1 wx

Mutant Starches, Single Mutants

Waxy (wx)

Deficiency of granule-bond starch synthase
Identified in maize, sorghum, rice, barley, wheat, & potato
Kernels of wx are full
Starch and dry weight are equal to normal
Mutant wx is epistatic to other known mutants, e.g. multiple mutants containing wx has NO amylose
Dosage effect shown for Wx wx wx
Broad applications in the food and non-food industries
Mutant Starches, Single Mutants

Amylose-Extender (ae)
- Deficiency of starch branching enzyme IIb (SBEIIb) for maize
- Identified in maize, rice, peas, & barley
- Kernels of ae have a smaller size than normal
- Dosage effect exists for ae gene
- Amylose concentration usually ranges 50-75% by blue value tests. Chromatographic analysis shows lower amylose value and the presence of intermediate materials
- Granules are smaller than normal, and some may be non-birefringent
- B-type x-ray pattern is shown for ae starch
- Broad applications

Sugary-1 (su1)
- Deficiency of starch debranching enzyme SU1
- Identified in maize, sorghum, & rice
- Standard sweet corn is su1 homozygous
- Kernels of su1 accumulates phytoglycogen >25% of kernel dry weight. Small starch granules may be isolated with amylose content possibly higher than normal
- Kernel development shows a pattern of:
  - Starch granules formed → granules partially degraded and replaced by phytoglycogen → granules mostly degraded and replaced by phytoglycogen
- Kernels have intermediate particles ranging from amylopectin to phytoglycogen

Sugary-2 (su2)
- Possible deficiency of a starch synthase SSIIa
- Identified in maize, possible in sorghum & rice
- Kernel dry weight is often reduced
- Starch granules have extensive internal fractures
- Amylose content is 10-15% higher than normal. Amylopectin has increased proportion of short chains
- Thermal properties by DSC are related to su2 dosage
Mutant Starches, Single Mutants

**Dull-1 (du1)**
- Deficiency of a starch synthase SSIII
- Kernel ranging from full size to semi-collapsed
- Average granule size smaller than normal
- Amylose content 5-10% higher than normal. Amylopectin has increased proportion of short chains
- Mutant du1 in sweet corn background may accumulate phytoglycogen

Mutant Starches, Multiple Mutants

**Amylose-Extender Waxy (ae wx)**
- Deficiency of both SBEIIb and GBSSI
- Reduced in size, dry weight, and starch content (~50%)
- Blue value test indicates 15-26% amylose, but chromatographic separation indicates solely amylopectin
- Both ae and wx functions independently
- Amylopectin has increased proportion of long chains
- Dosage effect exists for both ae and wx genes

**Amylose-Extender Sugary-1 (ae su1)**
- Deficiency of both SBEIIb and SU1
- Kernels not as full as ae, but fuller than su1. Weight and starch concentration are lower than normal
- Increased doses of ae results in reduced phytoglycogen
- Blue value test indicates 51-60% amylose content
- In su1, the initially formed starch granules are broken down and may be utilized to produce phytoglycogen. In ae su1, ae interferes with excessive branching and starch granules are formed along with small amount of phytoglycogen
**Mutant Starches, Multiple Mutants**

**Dull-1 Waxy (du1 wx)**

- Deficiency of both SSIII and GBSSI
- Kernels weight similar to du1 and wx, slightly less than normal
- Sugar concentrations are higher, and starch concentration is lower than in either normal, du1, or wx
- Gene wx is epistatic to du1, so du1 wx has 100% of amylopectin
- Amylopectin has increased portion of shorter chains

**Mutant Starches, Multiple Mutants**

**Amylose-Extender Dull-1 Waxy (ae du1 wx)**

- Deficiency of SBEIIb, SSIII, and GBSSI
- Starch concentration is low compared with the component single and double mutants. Sugar contents are several fold higher. Sweetness is between standard sweet corn (su1) and sh2 mutation.
- Amylopectin branching is different from either ae wx or du1 wx, and somewhat intermediate between wx and du1 wx

**Mutant Starches, Multiple Mutants**

**Amylopectin of mutants containing ae, wx, and du1**

Chain length profile of amylopectin

Chain length profile of β-limit dextrin
**Mutant Starches, the Potential**

Potential of mutant combinations

### Single mutants
- Waxy (wx)
- Amylose-extender (ae)
- sbel
- sbel2a
- Sugary-1 (su1)
- Zpr1
- Sugary-2 (su2)
- Dull-1 (du1)
- Brittle-1 (bt1)
- Brittle-2 (bt2)
- Shrunken-1 (sh1)
- Shrunken-2 (sh2)

### Double mutants
- ae wx
- sbel wx
- ae su1
- ae su2
- ae du1
- ae su1 su2
- de su2
- de su1 su2
- su1 su2

### Triple/quadruple mutants
- ae du1 su1
- ae du1 su2
- ae du1 wx
- su1 su2
- su1 su2 wx

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**Mutant Starches, Patents**

A list of mutant starch patents in last 20 years

- 2004, Method of grain production for heterozygous waxy sugary-2 maize
- 1996, Foodstuffs containing a waxy amylose extender starch
- 1992, ae du1 batter starch for deep fat fried food
- 1989, Foodstuffs containing starch of a waxy shrunken-2 genotype
- 1989, Foodstuffs containing starch of an amylose extender sugary-2 genotype
- 1988, Food stuffs containing starch of a dull sugary-2 genotype
- 1988, Food stuffs containing starch of an amylose extender dull genotype
- 1988, Food stuffs containing starch of a dull waxy genotype
- 1988, Starch of the duh genotype and products produced therefrom
- 1988, Starch of the wx fl1 genotype and products produced therefrom
- 1988, Starch of the wx sh1 genotype and products produced therefrom
- 1986, Bread containing wx su2 genotype starch as an anti-stulent

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**Mutant Starches, Patents**

An example:

Double Mutant Homozygous wx Heterozygous su2

Freeze-thaw stability of different starches (US patent # 6,828,474)

<table>
<thead>
<tr>
<th>Starch</th>
<th>Opacity</th>
<th>Gelling</th>
<th>Syneresis (Surface)</th>
<th>Syneresis (Pressed)</th>
<th>Overall stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Waxy</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2 doses</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

- Freeze-thaw stability: starch pastes were placed at -15°C for 16 hours and then thawed for 8 hours at room temperature. The samples were visually inspected for opacity, gelling, and syneresis (both on the surface and when pressed).
- Sample A: blend of approximately 10% waxy maize and 90% waxy maize with two doses of su2 gene
- Sample B: blend of approximately 20% waxy maize and 80% waxy maize with two doses of su2 gene
- 2 doses: pure waxy maize hybrid with two doses of su2 gene
- Each number represents the number of freeze-thaw cycles the starch paste remained acceptable (stable).
Genetic Modifications, The Challenges

Our strategy to generate novel starches

- Starch functionalities desirable by industries
- Starch structure required for desirable functionalities
- Starch materials for chemical & enzymatic modifications
- Methodologies connecting plant genetics with carbohydrate chemistry
- Manipulations of mutants & genotypes
- Mutations naturally occurred or artificially-introduced

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Genetic Modifications, Our Approaches

To identify and screen novel starches from seeds

Single kernel sampling (SKS) for starch analysis

- A methodology is established to screen novel starches from individual maize kernels while maintaining the vitality of each kernel
- SKS starch analysis may be conducted with high throughput when FACE (fluorophore-assisted carbohydrate electrophoresis) is used
- SKS may allow us to screen starch mutants from a large pool of seeds collected from natural plants or generated using artificially-induced mutagenesis

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Genetic Modifications, Our Approaches

Single kernel sampling (SKS) for starch analysis

- Analyzing starch structure while maintaining kernel vitality
- Potential high throughput endosperm starch screening

Genetic Modifications, Our Approaches

Roadmap to effective genetic starch modifications

- For selected genotypes, characterize the activity profiles of starch biosynthetic enzymes and starch structure (e.g. amylopectin branching pattern)
- Correlate enzyme activity profile with starch structure, elucidate their relationships, and construct a database
- For a starch structure required by a desirable function, propose feasible enzyme activity profiles based on the database and certain hypotheses
- Create genotypes rendering proposed enzyme activity profiles, AND, without sacrificing starch yield

Starch Analysis

Starch Fine Structure Analysis

Outline

- Why study starch fine structure?
- Fine structure and chain length distribution
  - Introduction of molecular weight
  - Molecular weight, degree of polymerization, and chain length
  - Polydispersity
  - Number average molecular weight
  - Weight average molecular weight
- Starch chain length distribution
  - Chain length distribution of starch
  - Methods to characterize chain length distribution
    - Size exclusion chromatography (SEC and HPSEC)
    - Fluorophore-assisted carbohydrate electrophoresis (FACE)
    - High performance anion exchange chromatography (HPAEC)
    - Comparisons
  - Structural parameters from chain length distribution
- Our capability to conduct carbohydrate analysis
Why study “starch fine structure”?

- To differentiate starches (and starch derivatives) at molecular levels
- To define and document unique starches or starch derivatives (e.g. maltodextrins) in both research reports and patents
- To monitor product profiles and maintain product consistency
- To control important properties (digestibility, retrogradation, solubility, hazing, water adsorption, viscosity, gel strength, stability, etc) of starches and starch derivatives
- To define goals for starch modifications
- To guide new product development

Starch fine structure is usually characterized by chain length distribution using cluster model

- Starch materials
  - Specific treatments
  - Completely debranched
  - Chain length distribution characterized
  - Structural parameters constructed

Chains are non-randomly clustered

Thompson, Carbohydrate Polymers, 2000, 43: 223-239

Chain length distribution is based on several basic concepts of molecular weight of polymers

- Molecular weight and degree of polymerization
- Polydispersity
- Number average molecular weight
- Weight average molecular weight
Molecular weight, degree of polymerization, and chain length

- **Molecular weight (MW):**
  - The mass of one molecule of the substance, relative to the “unified atomic mass unit” (equal to 1/12 the mass of one atom of carbon-12)

- **Degree of polymerization (DP):**
  - The number of monomeric residues in a polymer molecule

  “Chain length”, expressed in DP, is used to quantify molecular weight of starch chains

Polydisperse and polydispersity

This collection of molecules is polydisperse

Polydispersity index (PDI):

- Ratio of the weight average molecular weight to the number average molecular weight
- Indicating the overall distribution of individual molecular weight in a batch of polymers
- PDI equal to 1 indicates only one length of polymer is present (e.g. crystallized protein)
- In polysaccharides (e.g. starch), PDI may vary significantly.

Number average molecular weight ($M_n$)

- $M_n = \Sigma N_i M_i / \Sigma N_i$, where $N_i$ is the number of molecules of molecular weight $M_i$
- Can be determined by osmometry, end group titration, and colligative properties
- $M_n$ is more weighted by the small molecules in the molecular population
Weight average molecular weight ($M_w$)

- $M_w = \frac{\Sigma N_i M_i^2}{\Sigma N_i M_i}$, where $N_i$ is the number of molecules of molecular weight $M_i$

- Can be determined by light scattering, small angle neutron scattering (SANS), X-ray scattering, and sedimentation velocity

- $M_w$ is more weighted by the large molecules

Polydispersity index = $M_w / M_N$

An example of $M_w$, $M_N$, and polydispersity index

<table>
<thead>
<tr>
<th>$M_i$ (DP)</th>
<th>$N_i$</th>
<th>$N_i M_i$</th>
<th>$N_i (M_i)^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>10</td>
<td>1,000</td>
<td>100,000</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>100</td>
<td>1,000</td>
</tr>
<tr>
<td>$\Sigma$</td>
<td>20</td>
<td>1,100</td>
<td>101,000</td>
</tr>
</tbody>
</table>

$M_w = \frac{\Sigma N_i M_i^2}{\Sigma N_i M_i} = \frac{101,000}{1,100} = 91.8$ (DP)

$M_N = \frac{\Sigma N_i M_i}{\Sigma N_i} = \frac{1100}{20} = 55$ (DP)

So, polydispersity index = $\frac{91.8}{55} = 1.67$
Now, let us discuss chain length distribution

- Chain length distribution of following materials have been characterized
  - Starch (containing both amylose and amylopectin)
  - Isolated amylopectin
  - Beta-limit dextrin of amylopectin
  - Other starch derivatives, e.g. alpha-limit dextrin
- Then, but not always, the data of chain length distribution are used to calculate structural parameters of starch or AP clusters

Then, but not always, the data of chain length distribution are used to calculate structural parameters of starch or AP clusters.

These figures (by HPSEC) are called “chain length distribution (profile)”

MW, MN, and polydispersity index can be calculated from the curve

Usually, the chain length profiles are compared among different starches, to differentiate starches at molecular level

Starch molecules need to be debranched to release chains

A procedure to prepare debranched starch materials

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Starch extraction</td>
</tr>
<tr>
<td>2.</td>
<td>Dispersed in DMSO</td>
</tr>
<tr>
<td>3.</td>
<td>Adding buffer + isoamylase</td>
</tr>
<tr>
<td>4.</td>
<td>Incubation</td>
</tr>
<tr>
<td>5.</td>
<td>Moisture evaporated</td>
</tr>
<tr>
<td>6.</td>
<td>Debranched starch in DMSO</td>
</tr>
<tr>
<td>7.</td>
<td>Pullulanase needed to remove short stubs (DP2)</td>
</tr>
<tr>
<td>8.</td>
<td>Adding pullulanase</td>
</tr>
<tr>
<td>9.</td>
<td>Incubation</td>
</tr>
<tr>
<td>10.</td>
<td>Moisture evaporated</td>
</tr>
<tr>
<td>11.</td>
<td>Debranched BLD in DMSO</td>
</tr>
<tr>
<td>12.</td>
<td>Adding buffer + beta-amylase</td>
</tr>
<tr>
<td>13.</td>
<td>Incubation</td>
</tr>
<tr>
<td>14.</td>
<td>Moisture evaporated</td>
</tr>
<tr>
<td>15.</td>
<td>Debranched AP in DMSO</td>
</tr>
<tr>
<td>16.</td>
<td>Re-dispersion in DMSO</td>
</tr>
<tr>
<td>17.</td>
<td>Beta-limit dextrin (BLD) extraction</td>
</tr>
</tbody>
</table>

Waxy starches do not need AP isolation
Analysis of chain length distribution

- Three types of debranched starch material are usually prepared
  - Debranched starch
  - Debranched amylopectin
  - Debranched beta-limit dextrin

- Three types of separation methods are often used to describe the chain length distribution of these materials
  - Size exclusion chromatography (SEC or HPSEC)
  - Fluorophore-assisted carbohydrate electrophoresis (FACE)
  - Anion exchange chromatography (HPAEC)

Size exclusion chromatography (SEC or HPSEC)

- Eluent from HPSEC columns passes through a refractive index (RI) detector for quantifying the mass of carbohydrate molecules
- Molecular standards are used to calibrate columns and determine the molecular weight of samples
- Eluent may pass through a multiple angle laser light scattering (MALLS) detector for molecular weight determinations
Chain Length Distribution

Fluorophore-assisted carbohydrate electrophoresis (FACE): labeling of oligosaccharide using 1-aminopyrene-3,6,8-trisulfonate (APTS)

- APTS adducts of carbohydrate molecules has 2 properties
  - The APTS adducts are negatively charged, so may migrate in an electric field of electrophoresis
  - The APTS adducts may release detectable fluorescent emission with laser excitation at 488 nm
Molecular weight is determined by migration time
Number of molecules is determined by fluorescent signal

Longer migration time due to larger CHO molecule
Weaker fluorescent signal due to fewer molecules

Shorter migration time due to smaller CHO molecule
Stronger fluorescent signal due to more molecules

FACE conducted using capillary electrophoresis with laser-induced fluorescence

Electrophoregram of FACE

Courtesy of Beckman-Coulter
Comparison between HPSEC and FACE

High performance anion-exchange chromatography equipped with pulsed amperometric detector (HPAEC-PAD)

The adsorption/desorption equilibrium is governed by:
- The amount of negative charge of sample molecules
- The ionic strength of mobile phase

The greater the net charge, the stronger the adsorption
The greater the net charge, the higher the salt concentration required for desorption

Courtesy of Amersham Biosciences
The retention time of a molecule is determined by:
- Its net negative charge, which may be affected by pH
- Elution power, which is controlled by gradient elution

Carbohydrates can be separated via anion-exchange:
- In basic solution (high pH), carbohydrates are negatively charged.
- Thus, there exists an adsorption/desorption equilibrium of carbohydrate molecules with stationary phase.
- The higher DP of carbohydrate molecule, the greater net negative charge it possesses, the higher ionic strength (salt concentration) needed to desorb the molecule.
- Using gradient eluent, carbohydrate molecules with different DP are eluted at different retention time and separated.

PAD catalyzes the electrooxidation of carbohydrate molecules in high pH solutions, and gives signals proportional to the amount of molecules.

Typical waveform for PAD in alkaline solution at a gold working electrode.

(Courtesy of Dionex Corporation)
Chain Length Distribution

HPAEC-PAD gives a chromatogram similar to FACE

Pros and cons of HPSEC, FACE, and HPAEC

- HPSEC provides information of a broad range of molecular weight (>DP10,000) but with relatively low resolution >DP5
- FACE provides baseline resolution up to DP100, but unable to (with current techniques) give a chain length profile with broader DP range
- HPAEC is similar to FACE

HPSEC and FACE (possible HPAEC) are complementary

We have successfully demonstrated the compatibility of HPSEC and FACE in starch analysis, and established an HPSEC-FACE methodology balancing both high resolution and broad DP range

Yao et al, 2005, Carbohydrate Research, 340: 701-710
OK, we got chain length distribution, then what?

How to calculate structural parameters of starch or amylopectin?

- Number average chain length
- Average external chain length
- Distribution of external chain length
- Average internal chain length
- Distribution of internal chain length
- Average number of branches per cluster
- Average cluster repeating distance
- Distance of adjacent branches
- Chain length distribution of B2 chains
- Chain length distribution of B3 chains
- Chain length distribution of long B1 chains
- Chain length distribution of short B1 chains

Decoding chain length distributions

Yeo et al. 2004, Plant Physiology, 136: 3515–3523
**Capability of Carbohydrate Analysis**

- **What do we have?**
  - Beckman P/ACE MDQ capillary electrophoresis with LIF to conduct FACE
  - Waters HPLC system to conduct HPSEC and many other separations
  - Dionex HPAEC for sugar and starch analysis
  - Other systems

- **What can we do?**
  - Analysis of starch structure
  - Analysis of starch derivatives and hydrolysates
  - Analysis of simple sugars, oligosaccharides, and polysaccharides

- **Throughput?**
  - FACE up to 50~80 samples/day
  - HPSEC up to 10~20 samples/day
  - HPAEC up to 10 samples/day

- **Beyond the throughput?**
  - Methodology development
  - Product characterization
  - Optimize & define industrial process
  - And much more......

**Functional Properties of Starch**

- **Thermal properties**
  - Gelatinization
  - Swelling power
  - Retrogradation
  - Glass transition temperature

- **Rheological properties**
  - Brabender
  - RVA (rapid visco analyzer)
  - Viscoelasticity

**Thermal Properties**

- Starch gelatinization

- Isolated starch is in granular form, insoluble in water
- When put in cold water, the granules may absorb limited amount of water
- Generally, when temperature is below a certain value (for most around 60°C), the swelling is reversible
Thermal Properties

Starch gelatinization

- With higher temperatures (usually >65°C) irreversible swelling of starch granules may occur. "Gelatinization" begins.
- The exact temperature dependent on the specific starch, e.g., plant species, mutants, cultivars, granule sizes, etc.

Thermal Properties

Starch gelatinization

- Gelatinization occurs in a range of temperature, which can be quantified by DSC.

Thermal Properties

Starch gelatinization

- Since the loss of birefringence occurs at the time of initial gelatinization, it is a good indicator of the initial gelatinization temperature of a given starch.
Thermal Properties

Starch gelatinization

- The gelatinization range refers to the temperature range over which all the granules are fully swollen.
- Increased temperature and shear may cause granule disruptions.
- Increased translucency and increased viscosity may be observed during gelatinization.

65°C 70°C 85°C

Swelling power of starch granules

- When an aqueous suspension of starch is gelatinized, the granules undergo irreversible swelling.
- The degree is a function of the starch type and the presence of any physical or chemical modification.
- The swelling power is a measure of the hydration capacity of the starch and is expressed as the weight of centrifuged swollen granules, divided by the weight of the original dry starch.

Starch retrogradation

- Irreversible insolubilization of starch paste with formation of a precipitate or gel depending on concentration.
- Also known as "set back".
- Retrogradation may be caused by amylase as in regular starches.
- Retrogradation may also be caused by amylopectin as in waxy starches.
- Adjacent linear chains of amylase or amylopectin form double helices via hydrogen bonds and further aggregates to form crystalline regions.
Thermal Properties

Starch retrogradation is associated with...

- Bread staling, primarily caused by amylpectin retrogradation
- Ageing of cooked rice and tortillas
- Syneresis of starch paste after freeze-thaw recycles
- Opaqueness development of starch paste/gel/dispersion
- And other “bad” things

So it’s a reason for starch substitution to make stabilized starch

Thermal Properties

Starch retrogradation is also associated with...

- Production of resistant starch ($1.5/lb)
- Preparation of slowly digesting starch
- Film formation by amylose
- And possibly other “good” things

So it’s a reason to intensify starch retrogradation for value-added products

Thermal Properties

Starch retrogradation studied using DSC

Liu and Thompson, 1998, Cereal Chemistry 75(8)
Thermal Properties

Starch retrogradation studied using DSC

Fig. 5. Retrogradation of 30% ααααx starch after initial heating to 160°C as a function of storage time. Labelling refers to storage times.

Liu and Thompson, 1998, Cereal Chemistry 75 (6)

Thermal Properties

Starch retrogradation studied using DSC

Fig. 6. Typical thermograms of retrogradation for 30% ααααx starch with different initial heating temperatures and storage for 30 days at 4°C. Labelling refers to initial heating temperature.

Liu and Thompson, 1998, Cereal Chemistry 75 (6)

Thermal Properties

Starch retrogradation studied using DSC

Fig. 7. Retrogradation of 30% ααααx starch after initial heating to 85°C as a function of storage time. Labelling refers to storage times.

Liu and Thompson, 1998, Cereal Chemistry 75 (6)
**Thermal Properties**

How to manipulate starch retrogradation???

- Chemical modifications, substitution (stabilization)
- Enzymatic modifications, e.g. using partial beta-amylolysis
- Genetic modifications, e.g. su2 wx double mutants

You may also add sugars, lipids, etc to affect retrogradation, but structure modifications are the most effective approaches.

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**Rheological Properties**

Brabender Viscograph

- Gelatinization of starch and materials containing starch
- Hot and cold viscosity
- Stability of thickening agents or binders
- Acid stability of starch
- Measurement of liquids, suspensions, pastes, etc.
Modified Food Starches

- Converted starches
  - Acid conversions
  - Oxidized starches
  - Pyroconversions or dextrinizations
- Cross-linked starches
  - Distarch phosphate
  - Distarch adipate
- Stabilized starches
  - Starch acetate
  - Starch phosphate
  - Starch sodium octenyl succinate
  - Hydroxypropylated starch
- Other modifications
  - Beta-amylase treatment to reduce retrogradation
  - Pregelatinization for cold water-swelling starch

Properties and applications of modified starches

<table>
<thead>
<tr>
<th>Process</th>
<th>Function/property</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid conversion</td>
<td>Viscosity lowering</td>
<td>Gum candies, formulated liquid foods</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Adhesion, gelling</td>
<td>Formulated foods, batters, gum confectionery</td>
</tr>
<tr>
<td>Dextrins</td>
<td>Binding, coating, encapsulation, high solubility</td>
<td>Confectionery, baking (gloss), flavorings, spices, oils</td>
</tr>
<tr>
<td>Cross-linking</td>
<td>Thickening, stabilizing, suspension, texturizing</td>
<td>Pie filling, breads, bakesries, puddings, infant foods, soups, salad dressings</td>
</tr>
<tr>
<td>Esterification/ Etherification</td>
<td>Stabilization, low temperature storage</td>
<td>Emulsions, soups, frozen foods</td>
</tr>
<tr>
<td>Pregelatinization</td>
<td>Cold water swelling</td>
<td>Premix</td>
</tr>
<tr>
<td>Dual modifications</td>
<td>Combinations of properties</td>
<td>As you can imagine...</td>
</tr>
</tbody>
</table>

Modified Food Starches

- Converted starches
  - To reduce the viscosity and swelling power and increase the concentration in the dispersions

  - Acid-thinned starch:
    - Starch suspension is treated with dilute acid at a temperature below the gelatinization point
    - Granular form of the starch is maintained and the reaction is ended by neutralization, filtration, and drying once the desired degree of conversion is reached
    - This results in a reduction in the average molecular size of the starch polymers. Acid-thinned starches tend to have a much lower hot viscosity than native starch and a strong tendency to gel when cooled.

  - Oxidized starch
    - Starch suspension is usually treated with sodium hypochlorite
    - Commercial oxidized starch is granular and is insoluble in cold water
    - It is characterized by a whiter color than native starch, increased paste clarity and a low, stable viscosity on storage of the paste

- Dextrin
  - Starch hydrolysis products obtained in a dry roasting process either using starch alone or with trace levels of acid catalyst
  - Dextrins are characterized by good solubility in water to give stable viscosities. Four types exist: White, Yellow, British Gums and Solution-stable Dextrins
Modified Food Starches

Cross-linked starch

- **Cross-linked (bonded) starch**
  - Starch is treated with bi-functional reagents so that a small number of the starch polymer chains are chemically linked by the cross-linking reagent
  - Cross-linking inhibits granule swelling on gelatinization and gives increased stability to acid, heat treatment, and shear forces
  - Cross-linking is widely used to prepare chemically-modified starches for the processed food industry

- **Distarch phosphate**
  - A starch cross-linked with a phosphate linkage, e.g. from reagents such as sodium trimetaphosphate or phosphorus oxychloride

- **Distarch adipate**
  - A starch cross-linked with an adipate linkage, from adipic acid
  - Most cross-linked food starches contain less than one crosslink per 1000 glucopyranosyl units

Modified Food Starches

Starch stabilization

- **Starch stabilization**
  - "stabilization" is sometimes used to indicate the presence of a monofunctional chemical substituent which has the effect of stabilizing paste viscosity
  - Stabilized modified starches may be hydroxypropyl or carboxymethyl starch ethers
  - The monofunctional substituents also can be phosphate or acetyl ester groups
  - Generally the D.S. (degree of substitution) of these starches is between 0.01 and 0.2. The substituent groups have the effect of providing steric hindrance to chain association which stabilizes viscosity by preventing possible retrogradation.

Modified Food Starches

Starch alkenyl succinate

- **Starch alkenyl succinate**
  - A chemically modified starch produced by treating starch with alkenyl succinic anhydride under controlled pH conditions
  - Commercial alkenyl succinic anhydride available for use in food is the octenyl form
  - These starches have lipophilic ("oil-loving") properties and are used in emulsions and encapsulation

- **Starch octenyl succinate**
  - Common name given to Starch n-Octenyl succinate
  - Made by treating starch with n-Octenyl succinic anhydride at pH 8-8.5
  - Anionic due to a carboxyl group and hydrophobic due to the C8 unsaturated alkene chain
  - Food uses include encapsulation of flavors and emulsion stabilization
Modified Food Starches

Pregelatinized starch
- A type of starch which has been gelatinized and dried by the manufacturer before sale to the customer in a powdered form
- Pregelatinized starch can be made by drum drying, spray drying, or extrusion from either native or modified starch
- Pregelatinized starch develop viscosity when dispersed in cold or warm water without the need for further heating
- Pre-gelatinized starch is also known as precooked starch, pregelled starch, instant starch, cold water soluble starch, or cold water swelling starch (CWS)
- The degree of granular integrity and particle size have a major influence on their properties, e.g. dispersion and texture

Starch Enzymes and Starch Digestibility

Starch hydrolases
- α-Amylase
  - Endoenzyme cleaves internal α-D-(1,4)-linkages of glucan chains of amylose and amylopectin
  - Converts starch + H₂O to maltodextrins or syrups comprising simple sugars and oligo or polysaccharides
  - Average extent of hydrolysis can be quantified using DE value (dextrose equivalent)
  - Products with DE<20 is defined as maltodextrin, DE>20 is defined as (corn) syrup
- β-Amylase
  - Exoenzyme cleaves two glucosidic units sequentially from non-reducing ends of glucan chains
  - Exhaustive β-amylolysis converts starch to maltose + β-limit dextrins
  - Partial β-amylolysis was used to shorten amylopectin external chains to retard retrogradation

Starch hydrolases
- Amyloglucosidase (glucoamylase)
  - Exoenzyme that digests ungelatinized, partially gelatinized, or fully gelatinized starch
  - Releasing glucose by hydrolyzing both α-D-(1,4) and α-D-(1,6)-linkages from non-reducing ends
  - Industrially converts starch or liquefied starch (after α-amylase hydrolysis) to dextrose (glucose)
- Debranching enzymes
  - Specifically hydrolyze the α-D-(1,6)-linkages of branched α-D-glucan, e.g. amylopectin, glycogen, and their hydrolysates
  - Isoamylase and pullulanase are debranching enzymes with different functional behaviors
  - Widely used in starch structure analysis
  - Widely used in producing dextrose and maltose
Starch Enzymes and Starch Digestibility

Starch digestion

- Starch is digested in the human body
  - Firstly by α-amylase (salivary and pancreatic)
  - Yields maltose, maltotriose, and α-dextrins
  - Maltose and maltotriose are hydrolyzed to glucose by maltase
  - α-Dextrin by α-dextrinase (intestinal brush border enzymes)
  - α-Dextrinase (glucoamylase) is found at the brush border of the small intestines. It hydrolyzes α-D-(1,4) and α-D-(1,6)-linkages to produce glucose for absorption
- Other carbohydrate-digesting enzymes
  - Sucrase & Lactase

Adapted from Dr. Hamaker’s lecture in Starch Short Course

Starch Enzymes and Starch Digestibility

Starch digestion

- Starch, maltodextrin, glucose, sucrose, lactose make up “net carbs”
- Fiber constituents are not digested by the body’s enzymes
- Starch undigested by host enzymes, or “resistant starch” is usually readily digested in the proximal colon by bacterial amylases, and is about 20% utilized as energy for the body

Adapted from Dr. Hamaker’s lecture in Starch Short Course

Starch Enzymes and Starch Digestibility

Resistant starch

- Resistant starch (RS)
  - Starch that is resistant to digestion by α-amylase
  - Defined as “the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals.”
  - While RS escapes digestion in the small intestine, it may be fermented in the large intestine by colonic microflora
- RS has been classified in four different categories
  - Type I, resulting from physical inaccessibility in intact tissues or other large particulate materials
  - Type II, resulting from the physical structure of the uncooked, native starch granules
  - Type III, resulting from the physical structure of retrograded starch molecules after the starch granules are gelatinized
  - Type IV, resulting from chemical modification (e.g. cross-linking) that interferes with the enzyme digestion.