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Native or Raw Starch Digestion: A Key Step in Energy Efficient Biorefining of Grain

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Improved molecular disassembly and depolymerization of grain starch to glucose are key to reducing energy use in the bioconversion of glucose to chemicals, ingredients, and fuels. In fuel ethanol production, these biorefining steps use 10−20% of the energy content of the fuel ethanol. The need to minimize energy use and to raise the net yield of energy can be met by replacing high-temperature, liquid-phase, enzymatic digestion with low temperature, solid-phase, enzymatic digestion. Also called cold hydrolysis, the approach is a step toward a "green" method for the production of fuel ethanol. There has been substantial prior and increased recent interest in this approach that is presented in this first review of the subject. We include incentives, developmental research, fundamental factors of raw starch digestion, and novel approaches in enzymology and processing. The discussion draws on resources found in enzymology, engineering, plant physiology, cereal chemistry, and kinetics.

KEYWORDS: Raw starch; solid starch; digestion; enzymes; starch structure; cold cook; cold hydrolysis; liquefaction; saccharification; fermentation

INTRODUCTION

Biorefining: Grain to Glucose. The disassembly and depolymerization of grain starch to glucose are the result of the hydrolysis of α-1,4- and α-1,6-linkages between glucose monomers. Acid hydrolysis was used for this from its discovery in 1813 at least until the 1970s. However, the dilute acid and 120−150 °C temperatures used in this process corroded equipment, formed undesirable byproducts, limited yield, and was costly (*1*−*5*).

High-temperature, liquid-phase enzymatic hydrolysis is now used for starch hydrolysis. The use of enzymes was enabled by the discovery of naturally occurring thermostable bacterial and fungal enzymes. One basic enzymatic hydrolysis configuration is a three-step sequence. In the first step, a 30% (by weight) slurry is cooked in the presence of α-amylase to 90−165 °C, cooled if necessary, held at 90 °C for 1−3 h, and then cooled further to 60 °C with the addition of glucoamylase. Both batch and continuous processes are in use (*6*).

Fermentation-Excess Energy Demand. To estimate the energy issues of current practice, we introduce a "fermentation-excess enthalpy" for the energy required to heat the grain or starch and suspending fluid above the fermentation temperature (30 °C) to the peak temperature for the cooking process (**Table 1**). The calculation shows the excess energy demand is about 10−20% of the fuel value of ethanol produced. Demand, as opposed to usage, identifies an upper limit to the energy that may be saved through adoption of the technology. The estimate accounts for neither plant-specific thermal conversion efficiencies or heat transfer efficiencies that would raise usage above the calculated demand nor recovered heat that would lower usage. Opportunities for heat recovery include (i) backset or reuse of heated water and (ii) transfer exchange of heat from hot cooking liquor to distillation stripping. However, demand ameliorated by these measures comes at the expense of increased capital for heat exchange and specialized equipment. New energy-efficient, distillation-alternative separation technologies may reduce the need for the waste heat from conventional cooking.

An energy-conserving alternative is to lower the starch-to-glucose processing temperature to that of the fermentation temperature. This is below the onset of gelatinization at, for example, 54 °C for wheat, 60 °C for potato, or 65 °C for maize (*7*). In this alternative, there is reduced or no fermentation-excess enthalpy and the net energy yield would increase (**Table 1**). This hydrolysis strategy is applied to native or raw starch that is produced by wet or dry milling in the case of cereal grains. As digestion continues, the original starch granule structure disintegrates, the average molecular weight decreases, the starch polymer fragments dissolve, and finally soluble glucose forms.

Thermally gelatinized and liquefied or solubilized starch can have a viscosity higher than that of the starch slurry by a factor of 20-fold and is difficult to pump and/or stir. Low-temperature liquefaction provides an additional nonthermal energy benefit stemming from the lower viscosity inherent to the solubilization of low molecular weight starch fragments. Reduced viscosity may also increase the capacity of equipment applied to the conversion (*6*). Furthermore, the absence of high-temperature

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Table 1. Fermentation-Excess Energy Demand for Cooking[a]

peak temp (°C)	fermentation-excess enthalpy ΔH BTU/gal ethanol	GJ/m^3	% of LHV	estimated NEV	estimated energy cost U.S. ¢/gal	U.S. $/m^3	annual domestic energy cost for 5 billion gal or 19 million m^3 production (U.S. $)
90	6708	1.869	8.8	1.49	2.6–5.2	6.9–13.7	130–260
100	7803	2.174	10.3	1.53	3.1–6.0	8.2–15.8	155–300
120	9991	2.784	13.1	1.60	4.0–7.7	10.6–20.3	200–385
140	12181	3.394	16.0	1.67	4.9–9.4	12.9–24.8	245–470
165	14920	4.157	19.6	1.78	6.0–11.5	15.8–30.4	300–575

[a] These calculations assume a fermentation temperature of 30 °C and cooking methods reported in the *Alcohol Handbook* (*149*). Cost estimates assume that all enthalpy is provided by natural gas and represent both expected "floor" and recent high prices of $4–7.68/10^6 BTU or $3.80–7.20/GJ (*150*). Neither thermal efficiencies nor heat recovery are included. Heat capacities and heat of gelatinization are for maize. The heated and cooked fluid is a 30% aqueous suspension of corn starch. The lower heating value LHV of ethanol is 76000 BTU/gal or 21.2 GJ/m^3. The NEV or net energy value is the LHV divided by the total production energy and uses a NEV of 1.32 for each base case (*151*, *152*). Scheller estimated values of ΔH for cooking to be 20000 BTU/gal ethanol or 5.57 m^3, but the high temperature was not specified (*153*). Day reported the energy for cooking (conditions not specified) to be 14400 Btu/gal ethanol or 4.01 GJ/m^3 (*154*).

cooking minimizes the formation of undesirable Maillard reaction byproducts that reduce yield (*8*).

APPROACHES TO TECHNICAL HYDROLYSIS PROCESSES

Biocatalytic depolymerization of raw starch has been used in some form for the production of alcohol for centuries. Microscopic evidence for raw, ungelatinized starch as a substrate for beverage alcohol has been found in pottery vessels of ancient Egypt (*9*, *10*). Scot's whisky worts are not pasteurized (*11*). The low-temperature hydrolysis alternative has been described as cold, raw starch, solid starch, noncooking, raw flour, unsterile, and nonconventional (*12*−*15*). Published and patented developmental research using raw starch digestion for dextrose or ethanol dates from World War II (*16*, *17*). Several cold hydrolysis, corn-to-ethanol dry-mill plants based on raw starch digestion are now operating (*18*). The technology has attracted international interest for its use with not only temperate climate grains but also tropical fruit and root crops. In the tropics, starch digestion is viewed as an efficient means of utilizing unstable starchy food crops for which preservation is impractical (*19*).

The developmental research processes are listed in **Table 2** approximately in top-to-bottom order of decreasing temperature. Process entries at 40 °C and below are performed simultaneously with fermentation and exploit energy-conserving conditions. Processes above 40 °C utilize temperatures within the range of 55−65 °C at which some thermal swelling and gelatinization of the starch would be expected. Although identifiable starch granules remain at these temperatures, energy reduction is compromised.

Raw starch digestion in these reports makes use of purified enzymes (*17*), partially purified enzymes (*12*, *20*), unpurified natural enzymes (*21*−*23*), enzyme(s) produced in situ by the fermenting organism (*24*), or enzymes produced in situ by live organisms coimmobilized with the fermenting organism (*25*). Covalent immobilization of barley α-amylase for liquefaction has been reported (*26*). Efforts at scales varying from liter-sized up to at least 300 kL in commercial applications are represented (*18*).

A number of issues have been identified in these studies. These include high enzyme requirements because of low enzyme activity and poor enzyme stability, incomplete conversion, enzyme inhibition by glucose and maltose, and the potential for contamination (*27*, *28*). The one published analysis of process economics using this approach reported high costs of enzyme (barley α-amylase). The enzyme cost (0.22 Can$/U.S. gal) was 70% of the raw material cost for digestion of wheat and was the highest cost after the raw material (*29*, *30*). The enzymatic hydrolysis of solid cellulose by cellulases also has a high enzyme cost. The enzyme cost for starch to ethanol conversion in a state-of-the-art dry mill has been reported to be 4.5¢ per gallon of ethanol (*31*).

FUNDAMENTALS OF RAW STARCH DIGESTION

The fundamental factors known to contribute to or limit the success of raw starch depolymerization are described below. The focus is on substrate molecular, nano-, and macroarchitectures as well as enzyme factors of intrinsic activity, stability, and inhibition. Practical implementation issues are also discussed. Resources for this analysis include cereal chemistry, plant physiology, enzymology, molecular biology, biochemical engineering, and chemical engineering. The goal is to define biological and quantitative strategies that facilitate comparison, selection, and implementation.

Raw Starch Degrading Enzymes. Starch digesting enzymes include α-amylases, glucoamylases, α-glucosidases, isoamylases, β-amylases, and maltogenic β-amylases (**Table 3**). There are two endo amylase families: (i) α-amylases that randomly hydrolyze α-1,4-linkages between adjacent glucose units in the amylose and amylopectin polymers to produce dextrins and (ii) isoamylases that hydrolyze α-1,6-linkages at branch points in amylopectin (*32*). Endo-amylase α-1,4-hydrolyis is restricted in the region of α-1,6-branching. A number of cyclodextrin glycosyltransferase (CGTase) enzymes also digest raw starch by hydrolyzing α-1,4-linkages while catalyzing production of cyclodextrins (*33*−*35*).

Exo-enzymes function by hydrolyzing the terminal or next-to-terminal linkage starting at the nonreducing end of the glucose polymer. Glucoamylases produce β-glucose in this way by hydrolyzing both α-1,4-linkages and α-1,6-linkages at a slower rate. Glucosidases yield α-glucose and hydrolyze only α-1,4-linkages. Finally, β-amylases and maltogenic α-amylases produce β-maltose and α-maltose, respectively, except when an α-1,6-linkage is encountered, at which point hydrolysis by these enzymes stops (*36*, *37*).

The action modalities described above are based on classical kinetics using soluble substrate models. However, product distributions produced by the amylases suggest a variety of possible mechanisms. For α-amylase, the possibilities are classical random attack (from the solution or multiple chain attack), preferred attack at unique or susceptible sites, repetitive attack (also called single chain attack) in which one substrate fragment remains attached to the enzyme, and multiple site attack. The particular modality depends on the structure and

Table 2. Selected Research and Developmental Studies of Raw Starch Digestion: Processes Include Those with Discrete Liquefaction or Liquefaction and Saccharification (D) or Integrated or Simultaneous Liquefaction, Saccharification, and Fermentation (S)[a]

temp (°C), pH	substrate	enzymes	scale	process	ref
60, pH 4–4.5	wheat starch	*Anoxybacillus contaminans* (4D α-amylase), *A. niger* GLA		D	*121*
60, pH 4.5	wheat	*A. awamori*	L	D	*155*
60	barley	constituitive barley diastase (Scotch whisky)	>KL	D	*11*
55, pH 5–7	starch	*Humicola griseus* var. *thermoidea*	NS	D	*156*, patent to Genencor
50–65, pH 5–7	starch	*H. griseus* var. *thermoidea*	NS	D	*157*, patent to A. E. Staley
45, pH 4.5	wheat starch, flour, debranned wheat	barley malt α-amylase	5.0 L	D	*29*
45, pH 5.2	wheat, raw and boiled	purified porcine pancreatic α-amylase; purified maltase *A. oryzae*	L	D	*17*
45, pH 4.5	wheat, wheat flour, wheat starch	barley, *Bacillus*, and *A. oryzae* α-amylase from commercial sources	0.5L	D/S	*12*
40–50, pH 4–6	starch	*Corticium rolfsii* AHU 9627		D	*158*, patent to Godo Shushei Co., Ltd.
35, pH 4.8	waxy maize	α-amylase (Termamyl) and glucoamylase (Spiritazyme)	20 L	D	*159*
30–50, pH 3–9.5	starch	*Chalara paradoxa*	NS	D	*160*, patent to National Food Res. Inst. Ministry of Agric., Japan
40, pH 3.5	sago	coculture of *A. niger* N-10 and *S. cerevisiae*	0.5L	S	*161*
35, pH 3.5	cassava, sweet potato	α-amylase, glucoamylase, pectinase, xylanase, CMCase, and protease in *A. niger*, *A. awamori* (no. 20) as koji from corn hulls	0.5L	S	*15*
30–45, pH 5–5.5	corn starch	*Bacillus mesentericus* and *B. subtilis*	NS	S	*3*, patent to Wallerstein Co.)
30–40	corn	commercial: acid fungal glucoamylase	1–300L	S	*18*, *127*, patent application to Broin
30–40, pH 5	corn	amylases in *Chalara paradoxa*	0.5–1 kL	S	*96*, *162*
30, pH 4.2	corn	glucoamylase in *A. niger* as koji grown on wheat bran and *A. niger* and kawamori	0.5 L	S	*23*, *163*
30, pH 4.8	corn	free glucoamylase enzyme mixture (*Rhizopus* sp.) with *S. cerevisiae*	120 kL	S	*13*
30	rice	coimmobilized culture: *A. awamori* and *Zymomonas mobiliz* or *Rhizopus japonicus* and *Z. mobilis*, or *A. awamori* with *R. japonica* and *Z. mobiliz*	0.5 L	S	*25*
28	corn	recombinant *Saccharomyces* producing glucoamylase from *Rhizopus*		S	*24*
25–35, pH 4.75	starch	*Rhizopus* sp.	L	S	*148*

[a] If the scale of experimentation is not described as is sometimes the case for patents, it is noted as NS.

length of the substrate, pH, temperature, presence of inhibitors, and origin of the enzyme. Cereal α-amylases randomly attack high molecular weight substrate and nonrandomly attack short dextrins. The smallest resistant dextrins are produced by cereal amylases when compared to dextrins produced by porcine, human, *Aspergillus* sp., or *Bacillus subtilis* enzymes (*38*−*40*). The parallels to solid-phase digestion have not been described.

Amylases capable of raw starch digestion are found throughout the animal and plant kingdom. Amylases from cereals such as maize, barley, wheat, rice, and sorghum have raw starch hydrolytic capabilities that are essential to the breakdown of stored carbohydrates for plant development. Many species produce multiple α-amylases: as many as 10 in rice and five in potato. Furthermore, there is a wide variation in amino acid sequence among amylases from different plants (*41*). A one in 10 frequency of raw-starch digestion was found in soil and compost-derived bacteria (*42*).

Enzymes that digest raw starch are listed in **Table 3**. More than 80 natural starch-digesting enzymes have been reported since 1972, and the rate of discovery has been constant during this period. Uncharacterized, extracellular enzyme mixtures or systems with the ability to digest raw starch are noted separately in **Table 4**. The entries in these tables include green plant and microbial sources. Each source may also produce multiple amylases, not all of which digest raw starch. Wheat, for instance, produces an α-amylase isoform early in germination that digests raw starch and an isoform late in germination that only digests soluble starch (*43*).

Unique, non-native amylases also have been recursively evolved specifically to digest raw starch at or near fermentation

Table 3. Enzymes Reported to Digest Raw Starch[a]

organism	type	source	RSA	binding/other	ref
Bacillus firmus	CGT				*33*
Bacillus macerans BE101	CGT				*35*
Klebsiella pneumoniae AS-22	CGT				*34*
Aspergillus awamori	GA				*164*
Aspergillus awamori var. *kawachi*	GA				*165*
Aspergillus awamori var. *kawachi* F-2035					*116*
Aspergillus cinnamomeus	GA				*100*
Aspergillus oryzae (I)	GA		no		*117*
Aspergillus oryzae	GA				*166*
Candida antarctica CBS 6678	GA				*167*
Chalara paradoxa	GA	pith of sago palm			*113*, *160*
Cladosporium gossypiicola	GA				*115*
Corticium rolfsii	GA	tomato stem			*168*
Endomycosis fibuligera	GA				*14*, *169*
Fusidium sp. BX-1	GA				*170*
Lentinus edodes (Berk) Sing.	GA				*171*
Pencillum oxalicum	GA				*172*
Rhizoctonia solani	GA				*173*
Rhizopus niveus	GA				*14*
Rhizopus sp. MB46	GA				*174*
Saccharomyocopsis fibuligera	GA		no	lacks binding domain	*118*, *119*
Schizophyllum commune	GA				*175*
Thermomucor indicae-seudaticae	GA				*98*
Mucor rouxianus	GAII				*176*
Aspergillus sp. K-27	GA	soil			*86*
Bacillus stearothermophilus	MA				*177*
Streptomyces hygroscopicus	MA				*178*
Streptomyces precox NA-273	MA				*81*, *82*
Bacillus circulans	M6				*102*, *206*
Anoxybacillus contaminans	α			4 domain with SBD similar to CGT	*121*
Aspergillus awamori KT-11	α				*112*
Aspergillus ficum	α				*179*
Aspergillus fumigatus K27	α				*88*
Aspergillus sp. K-27	α	soil			*86*
Bacillus licheniformis (Termamyl)	α				*109*
Bacillus sp. IMD-370	α		no	alkalinogenic, α-cyclodextrin does not inhibit digestion β-cyclodextrin increases digestion	*180*
Bacillus sp. TS-23	α			also digests cyclodextrin	*181*
Bacillus sp. B1018	α				*42*, *182*
Bacillus sp. IMD 434	α		no		*183*, *184*
Bacillus sp. IMD 435	α		no		*185*
Bacillus sp. WN11	α	hot spring			*186*
Bacillus stearothermophilus NCA 26	α		no	no starch-binding domain	*99*
Bacillus subtilis 65	α		no	inhibited α-cyclodextrin	*108*, *187*
Candida antarctica CBS 6678	α				*167*
Chalara paradoxa	α	pith of sago palm			*113*
Clostridium butyricum T-7				acidogenic (pH 5)	*188*
Cryptococcus sp. S-2	α			SEQ	*189*
Gibberella pulicaris	α				*190*
Hordeum vulgare (barley)	α				
Hordeum vulgare (barley evolved)	α				*48*
Lactobacillus amylovorus	α				*191*
Populus canadensis Moench ≪robusta≫	α			no soluble starch activity	*101*
Rhodopseudomonas gelatinosa T-14, T-20	α			photosynthetic, host produces molecular hydrogen	*192*
Streptomyces bovis	α			bovine rumen	*193*
Streptomyces limosus	α				*194*
Streptomyces precox NA-273	α				*81*
Thermomyces lanuginosus F_1	α	municipal compost			*195*
Aspergillus sp. GP-21	AMG	soil			*196*
Aspergillus carbonarius	β	casava tuber			*197*
Bacillus cereus	β				*198*
Bacillus no. 2718	β	soil			*199*
Clostriduim thermosulfurogenes	β				*200*

[a] α, α-amylase; AMG, amyloglucosidase; β, β-amylase; CGT, cycloamylose glucanotransferase; MA, maltogenic amylase; GA, glucoamylase; and M6, maltohexaohydrolase. RSA is raw starch adsorption: noted as no if so reported.

Table 4. Enzyme Systems Applied Generally as Broth Reported to Have the Ability to Digest Raw Starch

organism	source	ref
Acremonium sp.		*97*
Aspergillus awamori		*155*
Aspergillus niger	rotting cassava	*201*
Bacillus alvei	soil	*19*
Bacilllus circulans F-2		*102*
Bacillus firmus/lentus	potato sludge	*202*
Bacillus sp.	soil and compost	*42*
Chalara paradoxa	sago palm pith	*203*
Cytophaga sp.	soil	*204*
Pestalotiopsis funerea, neglecta		*27*, patent to Sankyo
Humicola griseus thermoidea		*156*
Nodulisporum sp.		*97*
Synnematous sp.		*97*
Thermoactinomyces thalpophilus	soil	*205*

temperatures. Although represented as a single entry in **Table 3**, seven highly active and approximately 40 other variants have been isolated with the ability to digest raw starch. On the basis of barley α-amylase, these enzymes were created using biological combinatorial processes. They have the potential for overcoming limitations of the current natural set of enzymes. The tailored enzymes are created by random mutagenesis, expressed in *Saccharomyces cerevisiae* or *Escherichia coli*, and screened for digestion of soluble and/or native starch at 37 °C. The approach is to develop and explore sequence "space" for new molecular structure and function in the context of a single expression system. This sequence space is created by in vitro selection for activity under specific process conditions, rather than by natural evolution for biological function and survival. No assumptions were made about desirable molecular structure. New enzymes have been produced with 20 times increased specific activity and 1000 times increased total activity relative to the parent barley α-amylase expressed in the same host system. A sequence of screening based on soluble starch, dye-cross-linked soluble starch, and raw starch was used. From a group of enzyme variants with generally high activities, populations were found with high protein-specific raw starch activity and relatively low total activity as well as low specific and high total activity. Studies to elucidate these differences were not undertaken. A possible explanation is that molecular features favoring high expression may not favor reaction. It is also possible that the concentrations of enzyme in the screen may be close to saturating the available reaction sites or that the enzymes interfere with each other at high expression levels (*44*−*49*).

Enzyme Selection. The identification of the most promising candidate enzymes from the set of known starch-digesting candidates and the identification of their controlling genes will ultimately result in their transfer to and expression in a suitable fermentative or expression system. However, few of the identified natural enzymes have been applied in developmental studies. The best candidate identification would depend on superior kinetic capabilities of the enzyme: intrinsic activity, stability, inhibition, thermal stability, pH stability, etc. Comparisons are ambiguous because different starch substrates are used, and these are generally inadequately described at physical and molecular levels. Further kinetic parameters are either not determined or are mass-based or "apparent" kinetic parameters. These issues are described below.

Chemical and Physical Starch Architecture. Raw starches are granules that are spherical, polyhedral, or lenticular. Starches from a single botanic source will have unimodal or bimodal

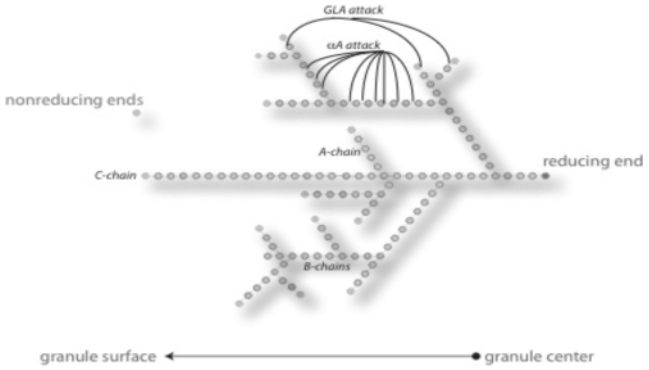

Figure 1. Simple model of starch structure illustrating descriptive chain nomenclature (see text) and classical enzyme attack modes by α-amylase (aA) and glucoamylase (GLA).

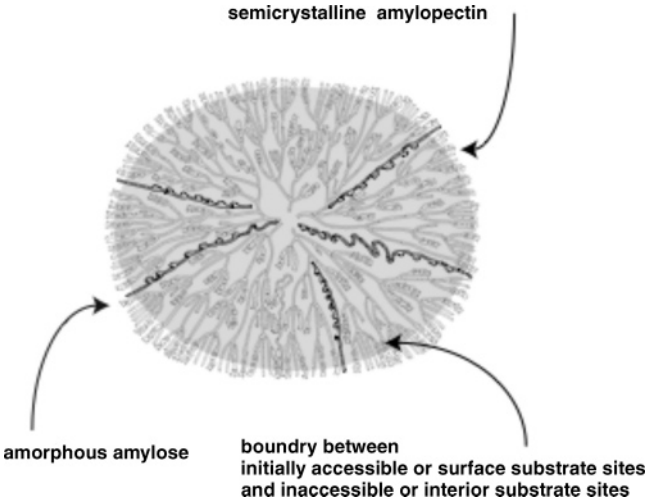

Figure 2. Conceptual model of starch granule structure that illustrates single helical organization of amylose and double-helical organization and grouping of amylopectin. Real boundaries may have a "hairy" appearance like that of a "hairy billiard ball" (*55*–*57*, *77*).

distributions (barley, rye, and wheat). Sizes decrease from potato (<110 μm) to wheat (<30 μm), corn (<25 μm), and rice (<20 μm) (*50*−*52*). Most starches are triphasic with concentric alternating growth rings or lamella of amorphous and semicrystalline character (*53*) as well as lipid amylose inclusion complexes (*54*). The structure of the nonlipid phase is due to the organization of amylopectin whose chains and branches pass through the lamella. The molecular structure grows from a single backbone or "C" chain containing the sole reducing group near the granule center or hilum and leads to a radiating cluster of parallel chains ("A" and "B" chains) connected together by α-1,6-branches (*50*, *51*). A chains are connected once, while B chains are branched and connected to two or more other chains (**Figure 1**). This led to an idealized model of starch structure that illustrates single helices of amylose and double helices and clustering of amylopectin (**Figure 2**). Although shown with a smooth exterior, the surface was hypothesized to have protruding branches of amylopectin. This was known as the "hairy billiard ball" model (*55*−*57*).

Long-range, bulk structural order due to the packing of double helixes is revealed by X-ray diffraction patterns: the "A" X-ray pattern of cereal grains, taro, tapioca, and sweet potato: the "B" X-ray pattern of tubers and high amylose maize; and the A and B combined X-ray pattern of legumes, roots, and some fruit and stem starches (*50*). Pea starch granules exhibit A X-ray pattern in outer regions and inner B X-ray patterns (*58*). The

patterns correlate with the length of the amylopectin branches (dp 23−29 for A type and dp 30−44 for B type). For B type starches, the α-1,6-branches are concentrated in the amorphous lamellae at the root of the chain clusters and the parallel glucans make up the crystalline lamellae. For A type starches, α-1,6-linkages are present in both amorphous and crystalline regions (*59*).

Short-range "surface" order unrelated to A and B X-ray patterns has been described using Fourier transform infrared spectroscopy (*60*). The degree of molecular order observed may correlate with digestive resistance. This molecular order is defined as the ratio of IR absorbances at 1045 and 1022 cm^{-1} and is believed to be associated with the structure of the α-helices. Amylose, a mostly unbranched component, is interspersed among amylopectin clusters in both the amorphous and the crystalline regions (*59*). However, tracking of gold-labeled pullanase and glucoamylase suggests increased compartmentalization of amylose and amylopectin for high amylose starches (*61*).

Cereal starches also contain lysophospholipids and free fatty acids at 0.5−1.0% dry weight (*62*). These lipids exist in either free form or as amylose−lipid inclusion complexes. The complex exhibits a "V" X-ray diffraction pattern, for the amylose chain in a helix with a cylindrical hydrophobic cavity (*63*). The cavity may include lipids, iodine, and surfactants and can be detected in solid-state NMR.

There are depressions on surfaces of dehydrated or wet starch granules that are seen by scanning electron microscopy. These are randomly distributed over granule surfaces (maize, sorghum) or clustered equatorially (wheat, barley) and are not seen on potato starch granules. The depressions may be architecturally enzyme-susceptible regions. Depressions are on the order of 1000 Å or 25× the amylase dimension (*64*−*70*). Surface roughness has been observed on wheat starch granules in the form of large projections, blocklets, or "blerbs" on potato granules. Peak-to-valley surface variation determined by atomic force microscopy (AFM) is on the order of 42 nm for wheat and 74 nm for potato (*71*). Surface depressions also have been observed using noncontact AFM for barley, maize, waxy maize, rye, oats, rice, and wheat (*72*).

Starch structure has been described by a three-dimensional "blocklet" model. The model suggests organization of the starch granule accounting for macro-, nano-, and molecular ordering and diversity. Among the included structures are double helical amylopectin, spacer regions between the helices and branching/structural conformations, double helical amylose/α-glucan, single helical amorphous amylopectin, single helical amorphous amylose, amylopectin−amylose helices, cocomplexes, and amylose−lipid V type inclusions (*73*, *74*). This molecular presentation of raw starch substrate to enzyme catalysis suggests resistance to enzyme attack and the important initial role for digestion by exo-enzyme action.

Digestion of raw starch granules may follow several possible routes. These include (i) local or distributed digestion at surface pores, (ii) centripetal digestion along starch polymer chains, (iii) digestion at artifactual cracks (*75*), or (iv) diffusion through the starch structure to susceptible sites. Of these, enzyme diffusion without digestion through the granule ultrastructure is precluded because α-amylase (4 nm) or glucoamylase molecules (8−10 nm) are bigger than the largest pores by factors of 7 and 10, respectively (*76*). This is consistent with the blocklet model and suggests that the enzyme must initially create its own cavities if gaps or fissures are absent. Unequivocal extrapolation of the reported observations to technical processes must consider that (i) starch preparation for the enzymolysis may include solvent extraction to eliminate lipid effects, (ii) the sample preparation may introduce artifacts due to dehydration and or physical abrasion, (iii) visualization aids such as dyes, labeling, and coatings may interfere with enzyme activity, and (iv) the digesting enzymes may be poorly characterized and/or may be mixtures of exo- and endo-digesting enzymes and their isoforms. The interaction of the glucoamylase molecule and the ends of glucoamylase chains as double helices at the starch surface has been conceptually modeled (*77*).

The specific mode of enzyme attack depends on both the botanic origin of the starch granule and the enzyme(s) involved. For digestion of wheat starch granules, α-amylase attack appears less severe than that of glucoamylase and is restricted to equatorial regions. The relatively mild α-amylase activity was suggested to be the result of poor presentation of surface molecules to the enzyme and to the α-D(1−4) bond specificity or endo-action of the enzyme (*78*). A similar pattern occurs for barley starch and purified barley α-amylase (two isoforms) or α-glucosidase (two isoforms). Digestion rate differences between the isoforms were large (*79*).

Internal digestion of hydrated maize starch has been followed by electron microscopy utilizing immunogold-labeled α−amylase from *Bacillus licheniformis*. Specifically, α-amylase migrates from the surface to the center (centripetal hydrolysis) by following the routes identified above. Subsequently, the core was degraded by digestion moving outward (centrifugal hydrolysis) (*80*). As digestion advances, interior lamellae or structures are exposed and exaggerated by preferential digestion of the lamellae. However, in one instance, lamellae were not apparent after digestion of maize by the maltogenic amylase of *Streptomyces precox* N-A273 (*81*, *82*).

Amylose−lipid inclusion complexes inhibit amylose digestion by α-amylases and β-amylases and may be enriched at the surface (*54*, *83*−*85*). Lipid complexes may be more effectively digested by amylase and/or lipase enzymes produced by organisms adapted to these complexes as substrate (*86*).

Starch−Substrate Susceptibility. Raw cereal starches are more completely and rapidly hydrolyzed than those from tubers or roots when digested by single, purified enzymes. The extent of ultimate conversion by hydrolysis has been used to develop a qualitative, five-point susceptibility scale (*87*). However, the extent of hydrolysis also depends on enzyme type, enzyme concentration, the hydrolysis metric (solubilization, turbidity, glucose appearance, reducing sugar appearance, etc.), reaction time, temperature, and the presence or absence of inhibitors (*67*). No quantitative correlation has been made between the initial hydrolysis rate and the starch chemical, nano-, or macroarchitectural features beyond the observation that A X-ray types exhibited by cereals are usually more readily digested than B X-ray types from roots. The reverse is true for A and B X-ray starches of pea (*88*).

There are subgroups of susceptibility within botanically pure starch populations. Hence, not all starch granules are digested at the same time (*89*, *90*). By contrast, acid hydrolysis erodes all starch granules (wheat) simultaneously (*91*, *92*). Acid hydrolysis degrades amorphous before crystalline regions leading to a break in the hydrolysis pattern.

Kinetics of Raw Starch Digestion. The selection of the best raw-starch digesting enzyme from a set of candidates requires comparing kinetic capabilities of the enzymes. Intrinisic activity, stability, inhibition, thermal stability, and pH stability are important to consider. Starch concentrations are usually expressed in mass units when calculating kinetic "constants" like

those determined from double-reciprocal rate and concentration plots (*93*). Substrate differences stemming from botanical, physical, and chemical diversity of the substrate are included in the "constants"; therefore, the kinetic parameters obtained need to be noted as "apparent". Branching frequency, hence the number of substrate sites for glucoamylase digestion, is dependent on botanic origin (*94*). As examples of the substrate diversity, the concentrations of amylopectin may differ by more than a factor of 20 (comparing grains and potato) and within a given botanical type by up to a factor of 5.

Consideration of the physical structure suggests further that the initial substrate concentration (α-1,4-bonds for α-amylase and α-1,4-bonds at nonreducing ends for glucoamylases) is concentrated in the solid-phase portion of the reaction mixture while it is not readily available to enzyme attack. However, as substrate is digested, additional sites for the reaction are exposed. Hence, the representation of an initial substrate concentration is ambiguous.

Reported initial rate data comparing the ratio of initial hydrolysis rate of insoluble to soluble starch represent all possibilities. For digestion of the same botanical starch in the form of native granules or solubilized and swollen (by cooking and cooling), soluble starch digestion was faster by a factor as high as 40 (*17*, *95*−*99*). There are reports of raw starch digestion rates that are greater than soluble rates by a factor of 1.3 to ∞ (*100*, *101*).

Comparative evaluation of enzymes or commercial enzyme preparations for raw starch (sweet potato) digestion exhibits a rate ratio of fastest/slowest of 2.2 for five glucoamylases (*Aspergillus niger* and *Rhizopus sp*. and one from *Endomycopsis fibuligera*) (*14*). The enzymes from *Bacillus stearothermophilus* NCA 26 exhibited a rate 10 times greater digesting corn and wheat starch than that of the least active culture *Bacillus* amyloliquefaciens S23 (*99*). Digestion rate by α-amylase from *Bacillus circulans* F-2 was about two-thirds of porcine α-amylase or *Streptococcus bovis* α-amylase, but 10−15× greater when digesting potato starch (*102*). A relative activity order for the amylase systems of pancreatic > bacterial > malt > fungal sources has been reported for digestion of corn starch (*103*).

Cereal amylase isozymes from a single source have different abilities to digest raw starch granules (*104*). As noted above, wheat produces amylases with and without the ability to digest raw starch (*43*). Barley α-amylase I more efficiently digests large and small starch granules from normal and waxy barley than the more plentiful α-amylase II. These enzymes degraded small granules by surface erosion and the large granules by generation of pinholes and internal erosion (*89*).

Enzymatic Synergies. Several enzymatic synergies have been reported for native starch digestion. Most common are synergies by endo- and exo-acting amylases. These are best described using a soluble starch model. Whenever an α-amylase acts alone, each catalytic event reduces the number of its substrate sites as well as their local concentration. When glucoamylase or α-glucosidase acts alone, there is no change in the number of substrate sites until the amylose or amylopectin is digested to the last residue. However, when these amylases act in concert, each endo-catalytic event also increases the number of substrate sites for the exo-acting enzymes, leading to an enhanced rate of conversion (*105*). This view of kinetics has been mathematically modeled for soluble-starch digestion (*106*). Soluble-starch digestion mechanisms described above also probably apply to raw-starch digestion, particularly in the late stages of digestion when most of the starch is soluble. However, early in raw-starch digestion, some catalytic events by either enzyme also may lead to physical disintegration of structure and consequent exposure of new sites susceptible to either or both enzymes.

Exo- and endo-amylase, raw-starch digestion synergies, have been reported for α-amylase and glucoamylase (*86*, *107*−*113*), α-amylases and glucosidases (*79*), α-amylases and maltase (*17*), glucoamylase and pullulanase (*114*), β-amylase and pullulanase, amylase broth and isoamylase (*21*), and commercial enzyme mixtures (*105*). Synergies have been reported for two endo-amylases: salivary α-amylase and pullulanase (*114*). The pullulanse, acting on α-1,6-branches may expose new sites for α-amylase that previously had been inaccessible because of steric factors introduced by the branch.

Adsorption/Enzyme Effectiveness. Physical adsorption of enzymes is often reported for raw starch digestion, but its role is unclear. Some bacterial amylases digest raw starch but do not adsorb. Glucoamylases from *Rhizopus niveus* and *Aspergillus awamori* var. kawachi F-2035 adsorbed on raw starch, but the amount adsorbed did not correlate with the hydrolysis rate (*67*, *115*, *116*). Adsorption of *Aspergillus oryzae* glucoamylases was inversely correlated with raw starch digestion (*117*). The raw starch-digesting glucoamylase from *Saccharomycopsis fibuligera* has been found to lack the molecular region usually correlated with starch binding (*118*, *119*). Adsorption has been shown to be reversible for digestion of insoluble amylose by α-amylase and glucoamylase (*120*).

In general, larger amounts of enzyme are required for solid-starch digestion than for soluble starch digestion. In the study of botanical susceptibility of raw starches to glucoamylases, up to 200× the amount required for complete soluble starch digestion was applied (*67*). Digestion of wheat starch required 1000× the amount of barley α-amylase expected for soluble starch digestion, with 10^{10} enzyme molecules supplied per starch molecule (*12*). Under these conditions, enzyme cost was second only to raw material cost (*30*). Viewed on a cost basis, reduction of enzyme cost is needed to maintain energy-savings cost benefits of the raw-starch digestion strategy (*8*).

The need for higher enzyme concentrations in raw, as opposed to soluble, starch digestion may be due to the localization of the substrate to the surfaces of the granules but also to less-effective catalytic events. For catalysis in which the enzyme concentration is not saturated and for the case of surface localization, an increase in the concentration of enzyme might be expected for solid and soluble conversions to be equivalent since the surface blocks the approach of soluble enzymes from half of the solid site-surrounding space. However, in raw-starch digestion, it is probable that enzyme−substrate interactions include imperfect docking or binding events that do not result in catalysis. Both the molecular and the physical architecture of the solid substrate and the molecular configuration of the enzyme may contribute to this inefficiency (*60*, *121*). Furthermore, some interactions may lead to physical, irreversible, or noncatalytic binding. Hence, both productive and nonproductive interactions can be expected. High enzyme concentrations increase the frequency of productive interactions.

Contamination. A grain to ethanol processing strategy that employs uncooked, raw starch digestion is inherently susceptible to microbial contamination that may reduce ethanol yield, yeast crop yield, and carbohydrate utilization and may increase lactic acid and foam production. However, in a large-scale noncooking process for corn, low bacteria levels were reported (*13*). *Lactobacillus* sp. (*Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum*) have been implicated as major problems for ethanol production,

although reliable correlations between inoculum levels and reduction of ethanol yield have not been established (*11*, *122*−*124*).

Chemical, radiological, thermal, and antibiotic treatments may be invoked as noncooking processes to replace thermal pasteurization. Antibiotics are already routinely used in fuel alcohol fermentations (*125*−*127*). Refrigeration of saccharified wort is used in traditional Scot's whisky distilleries. Low pH processing using acid-tolerant enzymes has been reported for sake processes (*128*). Other methods include (i) γ-radiation for cane (*129*), (ii) γ-radiation for noncooked corn (*95*, *130*), (iii) chlorine dioxide and sodium metabisulfite for silage (*131*), and (iv) hop acids and other natural plant chemicals (*132*−*135*). Combined nonthermal methods have also been suggested (*136*).

Alternate methods of refining grains may reduce contamination issues. Some of these experimental prefermentation, fractionation, and concentration methods make use of aqueous ethanol as a process fluid/separation agent. The methods include "intensive" use of dry feedstock grain as a gas-phase, azeotropic ethanol-dehydrating adsorbent prior to its fermentation (*137*), simultaneous liquid-phase extraction of oil and protein from dried grain by ethanol and adsorption of water by the grain from azeotropic ethanol (*138*−*141*), extraction of prolamine protein (*142*, *143*), and cold ethanol wheat-starch displacement from hydrated dough (*144*−*147*).

Modifying processes so as to shorten digestion times and lower temperatures during liquefaction and saccharification utilizing advanced enzyme systems may help to limit contaminant growth in differentiated processes. Furthermore, in fully integrated processes, the substrate concentration for microbial growth will be minimized by matching the biocatalytic hydrolysis that produces sugars with the fermentation that consumes them (*8*, *148*).

In conclusion, significant reductions in the process energy for the conversion of grain to ethanol may be achieved through the use of raw starch digestion. Currently, slow or incomplete conversion, high enzyme requirement and cost, and the opportunity for contamination have impeded the adoption of this technological strategy. Enzyme discovery through mining of natural sources has already increased the spectrum of useful catalytic properties. Enzyme discovery through combinatorial and directed evolution has strong potential for overcoming existing catalytic and physical limitations and has already dramatically increased the available resources. Rigorous characterization of the enzyme resources on well-defined substrates is needed to allow rational comparison of alternate enzymes and enzyme systems. It may be possible and necessary to exploit and tailor enzyme synergies to account for botanical and architectural differences in starch structure. New processing approaches (i) employing ethanol in the preparation and separation of grains prior to fermentation and (ii) in simultaneous liquefaction, saccharification, and fermentation may help to minimize or eliminate contamination issues.

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