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A Destructive-Reconstructive Strategy to Engineer Low-Glycemic *Dioscorea Alata* Starch via Sequential Acid Hydrolysis and Retrogradation

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ABSTRACT

A "destructive-reconstructive" strategy employing dual treatments of controlled acid hydrolysis and thermal retrogradation was investigated to engineer a novel, low-glycemic water yam starch. The starch was partially hydrolyzed with 0.5N HCl for varying durations (0, 4, 10, and 18 h) and subsequently retrograded by alternating cold (4°C, 18 h) and ambient (30°C, 6 h) temperatures for 48 hours. This modification induced significant changes in the starch's physicochemical properties, including amylose content, molecular weight, and solubility, with FTIR analysis confirming the hydrolytic cleavage of starch chains into smaller fragments. A critical finding revealed that shorter hydrolysis times enhanced the formation of crystalline regions, thereby limiting subsequent acid and enzymatic degradation. Consequently, extending hydrolysis time systematically decreased the degree of relative crystallinity while concurrently increasing the in vivo glycemic index (iGI). The modified starches exhibited in vitro glycemic indices ranging from 35.25% to 49.23% and in vivo values from 51.07% to 64.67% relative to native starch. In vivo validation using a mouse model highlighted significant discrepancies from in vitro estimations, underscoring the necessity of physiological assessment for developing functional food ingredients. This dual modification demonstrates a promising approach for creating low-glycemic starches for individuals with diabetes and those on calorie-restricted diets.

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1. Introduction

The water yam (*Dioscorea alata*) is a tuberous plant species that serves as a vital food source and holds economic significance for people in several countries across Africa, the Caribbean, Asia, and the Americas. The *Dioscorea* genus includes over 600 species, but only a few are cultivated for food and medicinal purposes. *Dioscorea alata*, commonly known as water yam, is the most extensively cultivated yam variety in Vietnam. Its advantages for sustainable production include ease of propagation and cultivation, high nutritional value, and a relatively long shelf-life of the tubers. Reports indicate that dry water yam starch typically contains 9.2-10.3% moisture, 70.9-73.9% carbohydrates, and 8.4-10.5% protein. Additionally, it is rich in minerals such as sodium, potassium, calcium, magnesium, phosphorus, and iron. Water yam is suitable for producing modified starch due to its long average chain length, high degree of polymerization (DP), and relatively high crystallinity compared to corn, rice, and potato starch [1].

The glycemic index (GI) of food measures the rate at which glucose is released into the blood after consuming carbohydrate-rich foods. Controlling GI is crucial for individuals with weight issues, metabolic disorders, and diabetes [2]. Consequently, many studies focus on developing low-GI starch by modifying the starch structure.

Partial hydrolysis of potato starch with acid significantly alters its structure, reducing the average

particle size and mass. It was shown that partial hydrolysis and heat-moisture treatment of potato starch increased its ratio of alpha helix/amorphous structures, the resistant starch content and GI [3].

After starch undergoes gelatinization and then cools, the amylose and amylopectin chains, initially in an amorphous state, can reassociate and form a new ordered structure. This process, called starch retrogradation, changes various physicochemical properties, notably the starch's digestibility [4].

In the pursuit of creating resistant starch, two primary philosophical approaches exist. Pathway A, "Constructive Refinement", focuses on physical methods like thermal cycling [5] or annealing, which act on intact (albeit gelatinized) starch chains to enhance and perfect the formation of crystalline regions, typically leading to an *increase* in overall degree of relative crystallinity (DRC). The goal of this pathway is to maximize long-range order. Conversely, Pathway B, "Destructive-Reconstructive", is the concept investigated in this manuscript. This process involves an initial chemical degradation step (acid hydrolysis) to selectively cleave glycosidic bonds in the amorphous structure post-gelatinization [6]. This creates a population of shorter, more mobile glucan chains. The subsequent retrogradation stage is not about 'refining' an existing structure, but rather an attempt to 'build' an *entirely new* one from these fragments.

Based on this conceptual framework, we hypothesize that this two-stage "destructive-reconstructive" process will yield a unique starch architecture. The initial hydrolysis generates shorter chains which, despite their higher mobility, will be spatially hindered from forming the highly ordered, long-range crystalline structures seen in retrograded native starch. Instead, they will form a network of smaller, denser, yet imperfect microcrystallites. We further hypothesize that this unique architecture, characterized by a possibly *lower* overall DRC, may exhibit superior enzyme resistance and a lower glycemic index compared to retrograded native material, and that this effect is dependent on the initial degree of hydrolysis.

Recognizing the documented limitations and potential inaccuracies of purely *in vitro* glycemic index estimation methods [7], [8], this study incorporates a rigorous *in vivo* evaluation in a mouse model to provide a physiologically relevant measure of glycemic response, a critical step in validating the potential of these modified starches as functional food ingredients.

2. Materials and Methods

2.1. Starch isolation

Water yam starch was obtained from water yam tubers purchased at local markets. Water yam starch isolation was carried out according to the method described in previous studies [1], [9]. Starch was isolated from water yam tubers by washing, peeling, and cutting them into small pieces. The pieces were blended with distilled water to form a slurry, which was filtered to remove fibers. The obtained starch milk was then soaked in an 0.2% NaOH solution, then allowed to settle, the starch precipitate was collected, and the supernatant was repeatedly removed until no trace of protein remained (checked using Ninhydrin reagent). Afterward, the starch milk was neutralized and repeatedly washed with distilled water to remove impurities. Finally, the purified starch was dried at 40 °C and ground into a fine powder for storage.

2.2. Partly hydrolysis and retrogradation of starch

The native water yam starch was hydrolyzed with HCl using the method of Jayakody and Hoover with some modifications [10]. A native starch suspension (3%, 400 mL) was heated in a water bath at 95°C for 30 min. Then, the starch gel was further treated by autoclaving at 121°C for 15 min for complete gelatinization. After cooling to room temperature, the starch gel was supplemented with 96 mL of 0.5N HCl solution. To stop the reaction, the starch solution was neutralized with 1N NaOH and centrifuged at 8000 rcf for 20 min (Hermle Z366, Benchmark). The supernatant was used to determine the total carbohydrate content by the phenol-sulfuric acid method and the degree of hydrolysis (DH) according to Equation 1 [11], [12]:

$$DH\ (\%) = \frac{MR \times 0.9}{MT} \quad (1)$$

Where: MR (g) is the total carbohydrate mass in the supernatant and MT (g) is the mass of starch before hydrolysis. The residue was washed with deionized water (DW) and centrifuged (8000 rcf for 20 min, ×3 times) to completely remove NaCl (test by $AgNO_3$ solution).

The starch gel (residue) after centrifugation is dispersed in water to achieve a concentration of 60% (v/v) and subjected to two cycles of retrogradation, each cycle sequentially at 4°C for 18 h and 30°C for 6 h. Following retrogradation, the starch gel was centrifuged at 2400 rcf for 10 min to collect the residue. The collected residue was then spread thinly and convectively dried (50°C, 24h), ground, and sieved through a 120-mesh sieve [3]. The retrograded samples with hydrolysis times of 4, 10, and 18 h were designated as HR4, HR10, and HR18, respectively [9]. The control sample (designated as HR0) was prepared similarly, but with 96 mL of DW instead of 0.5N HCl solution in the hydrolysis step. In addition, native starch (A) was also used as a control sample.

2.3. Starch characterizations

Fourier-transform infrared (FTIR) spectra of the starch powders were recorded with an FT/IR 4700 spectrometer (Jasco, Japan) in the wavenumber range from 400 to 4000 cm^{-1} [13]. The spectra were recorded with a resolution of 2 cm^{-1}. For each measurement, approximately 0.1 g of the dry starch sample was firmly compressed onto the crystal of an Attenuated Total Reflectance (ATR) accessory.

The X-ray diffraction (XRD) spectra of the starch samples were analyzed using a D8 Advance Eco instrument (Brucker, Germany) equipped with a Cu–Kα radiation source at a wavelength of 0.15406 nm, and a scanning angle (2θ) of 3-30° with the voltage and current set at 40 kV and 40 mA, respectively. The degree of relative crystallinity (DRC) was calculated using the following Equation 2 [14]:

$$DRC\ (\%) = \frac{A_c}{A_c + A_a} \tag{2}$$

Where, A_c and A_a are the areas of the crystalline region and the amorphous region, respectively.

The reducing power of starches was determined using a previous method [15]. Besides, the apparent amylose content (AAM) was determined using the iodine-binding colorimetric method [16]. Briefly, 100 mg of starch was solubilized in a NaOH solution. An aliquot was then neutralized with HCl before reacting with an I_2/KI solution. After a 30-minute incubation, the absorbance of the resulting blue complex was measured at 620 nm and quantified against a pure potato amylose standard curve.

The intrinsic viscosity (η_i, mL/g) is a key parameter that reflects the structural characteristics, size, shape, and molecular weight of starch molecules, allowing for the estimation of their average molecular weight [17], [18]. For analysis, starch solutions with concentrations ranging from 1.0 to 5.0 mg per 1.0 mL of 1 M KOH were prepared. The kinematic viscosity (η, m^2/s) of these solutions was measured at 30°C using an Ostwald capillary viscometer (Ø = 0.3 mm, Ref. No 509 03, Germany). The relative viscosity (η_{rel}) was calculated as the ratio of the kinematic viscosity of the starch solution to that of the pure KOH solvent (η_o, m^2/s). This value was then used to compute the reduced viscosity (η_{red}) using Equation 3:

$$\eta_{red} = \frac{\eta_{rel} - 1}{c} \tag{3}$$

where c is the starch concentration (1-5 mg/l of 1M KOH). Based on these viscosity data, the intrinsic viscosity and the average molecular weight (M_w) of the starch samples were determined following established methodologies [19], [20].

The solubility (SB) and swelling power (SP) of the starch samples were determined using a method described in a published scientific paper [21]. Starch dispersions (1%, w/v) were heated for 30 minutes at various temperatures (25, 45, 65, and 85°C) with periodic vortexing. After centrifugation (2400 rcf, 20 min) to separate the sediment (m_2) and the supernatant, both fractions were dried to determine their respective solid weights (m_3 and m_4). Solubility and swelling power were calculated using equations 4 and 5:

$$SB\ (\%) = \frac{m_4}{m_1} \times 100 \tag{4}$$

$$SP\ (g.g^{-1}) = \frac{m_2 - m_3}{m_3} \quad (5)$$

where m_1 is the initial starch weight.

Paste clarity of starch was expressed as the percentage transmittance (%T). Starch suspensions were prepared at concentrations ranging from 1.0 to 5.0% (w/v), gelatinized (95°C, 30 min) with continuous stirring. The starch gels were then cooled to room temperature and the transmittance (%T) was measured at 640 nm using a UH5300 UV-Vis spectrophotometer (Hitachi, Japan) [22].

2.4. In vitro estimated glycemic indexes (eGI)

The eGI was determined using an enzymatic hydrolysis procedure adapted from the previous method [8]. Specifically, an α-amylase enzyme solution (AHA 400, Angel Yeast Co., Ltd.) with an activity of 10 U/mL was prepared in sodium phosphate buffer (pH 6.9). Starch samples (0.5 g) were dispersed in 25 mL of the buffer and equilibrated at 37°C. Hydrolysis was initiated by adding 5 mL of the enzyme solution. Aliquots were withdrawn at regular intervals, and the enzymatic reaction was terminated by adding 3,5-dinitrosalicylic acid (DNS) reagent.

The concentration of reducing sugars released (expressed as glucose equivalents) was quantified spectrophotometrically at 530 nm using a glucose standard curve. The hydrolysis curves, plotting glucose equivalents versus time, were fitted to a first-order kinetic model (equation 6) as proposed by a published study (Goñi et al., 1997) to determine kinetic parameters:

$$C = C_\infty\left(1 - e^{-kt}\right) \quad (6)$$

where, C is the glucose content at reaction time t, C∞ is the glucose concentration when the starch is completely hydrolyzed (C at time t_∞), k is the kinetic constant. The eGI of each sample was then calculated relative to the completely gelatinized native starch sample (A), which was used as the reference and assigned an eGI of 100.

2.5. In vivo glycemic indexes (iGI)

The iGI study was conducted on male mice (*Mus musculus* var Albino, purchased from Ho Chi Minh City Pasteur Institute) using the method described by a previous study [23]. Before the experiment, the mice were acclimatized to the laboratory environment (29±1°C, 12h light/dark cycle, with ad libitum access to water and a standard AIN-93 diet) until they reached an average weight of 30±1 g per individual. All procedures were approved by the Institutional Ethics Board at Dinh Tien Hoang Institute of Medicine (Certificate No. IRB-A-2301) and performed in accordance with national guidelines for the care and use of laboratory animals. Before the experiment, the mice (5 individuals/group) were fasted for 12 h, then administered a glucose or a starch solution (7%, w/v; 0.5 mL) via oral gavage. Blood was collected from the tail vein of each mouse via a small incision (tail snip technique) at pre-ingestion (0 minutes) and post-ingestion (15, 30, 60, 90, 120 and 180 min). Blood glucose levels were measured using the handheld Benecheck Plus blood glucose monitoring device (General Life Biotechnology, Taiwan). The blood glucose measurements were used to estimate iGI using the method of Wolever and Jenkins, with glucose considered as 100 [24].

2.6. Statistical analysis

All data were triplicated, and the results were reported as the Mean ± standard deviation. The analysis of variance (ANOVA) was conducted using the Duncan test using the SPSS software (Version 22) to evaluate the significant differences in the means ($p < 0.05$).

3. Results and Discussion

3.1. Reducing power (RP)

When starch was hydrolyzed by acid, the glycoside bonds of starch were randomly cleaved, thereby reducing the length of the starch chains. Amylose has one reducing end (with a -CHO group) and one non-reducing end, while each amylopectin molecule has only one reducing end and multiple non-reducing ends. The hydrolysis of starch results in the formation of numerous linear molecules, thereby increasing the reducing power of the starch sample.

It could be observed that the reducing end content of the gelatinized starch (A) is higher compared to the native sample (Table 1). This result might be attributed to the depolymerization of starch molecules during heat treatment. (autoclave 121°C/15 minutes) [6]. With increased hydrolysis time, more short chains were formed, and these chains were more soluble in water than longer chains.

3.2. Apparent amylose content (AAM)

When interacting with iodine, amylose forms a characteristic blue complex. In contrast, amylopectin, with branched structure, reacts with a small amount of iodine to produce a reddish-brown or purple complex [16]. Figure 1a illustrated the changes in the absorption intensity of the starch-iodine complex at various degrees of hydrolysis. The results indicated that different hydrolysis times have influenced this complex-forming ability of modified starch. Additionally, the results revealed differences in the shape of the absorption curve and the absorption peak between native and modified starches, but there was not much difference when comparing different modified samples with each other.

The starch-iodine complex exhibits an absorption peak (λ_{max}) at 620 nm (deep blue), while the absorption peak for the pure starch-iodine complex is at 618 nm. Experimental results (Table 1) showed that the absorption peak of native starch was 598 nm. Meanwhile, the maximum absorption peaks of modified starches were lower than the native but tended to shift towards peaks from 560 nm (HR0) to 565 nm (HR18) (Figure 1a and Table 1). These peaks nearly corresponded to the wavelengths of amylopectin (560 nm and 728 nm) [16].

Table 1 also demonstrated that following gelatinization, partial hydrolysis and retrogradation of starch, degree of hydrolysis (DH) gradually increased with reaction time, accompanied by a slight increase in the wavelength of the absorbance peak and its absorbance. Moreover, compared to native starch (N), the modified starch samples (HRs) exhibited significantly lower AAM (apparent amylose content), indicating that these samples underwent substantial degradation. Sufficiently long amylose segments participated in the formation of the α-helix structure and crystalline structure of starch. These structures reduced the complexation ability of amylose with iodine molecules [4]. As observed, AAM gradually increased from 0.06 to 1.59% with the prolonged hydrolysis time. This outcome was resulted in the random effect of acid during hydrolysis, which cleaved α-1,4- and α-1,6- glycoside bonds, depolymerized amylopectin branches, and reduced amylose chain length. However, prolonged hydrolysis generated many shorter chain segments, which hindered the formation of alpha helix structures, consequently leading to an increase in AAM with extended hydrolysis time [6].

3.3. Viscosity and average molecular weight (Mw)

Intrinsic viscosity (η_i) can be employed to estimate the average molecular weight (M_w) of starch samples [18]. Following experimental procedures, the linear regression equation of reduced viscosity (η_{red}) was derived (Figure 1b). Indirect calculation of intrinsic viscosity (η_i) and average molecular weight (M_w) was achieved based on these linear regression equations (Figure 1b), and the results were presented in Table 1.

Table 1. *Reducing power (RP), Degree of Hydrolysis (DH), wavelength at peak, absorbance at peak, apparent amylose content (AAM), Intrinsic viscosity (ηi) and average molecular weight (Mw) of starches.*

Sample	RP (mg/g)	DH (%)	Peak wavelength (nm)	Peak absorbance	AAM (%)	η_i (mL/g)	M_w ($\times 10^5$ g/mol)
N	3.79±0.10^a	0	598	0.30	17.25±0.02^e	264.34±0.02^e	10.27±0.05^e
HR0	31.63±0.43^c	6.76±0.05^a	560	0.17	0.06±0.03^a	258.19±0.01^d	10.00±0.02^d
HR4	42.51±0.11^d	10.26±0.02^b	560	0.18	0.53±0.01^b	243.85±0.04^c	9.38±0.01^c
HR10	49.28±0.20^e	13.35±0.03^c	564	0.18	0.73±0.02^c	233.61±0.02^b	8.94±0.03^b
HR18	56.45±0.01^f	18.31±0.05^d	565	0.19	1.59±0.02^d	165.99±0.02^a	6.09±0.02^a

Within each column, statistically significant differences ($p<0.05$) are indicated by different superscript characters.

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Based on the findings presented in Figure 1b, the native starch (N) exhibited the highest reduced viscosity (η_{red}) compared to the modified samples. The reduced viscosity of the modified samples decreased gradually with increasing hydrolysis levels (HR0>HR4>HR10>HR18). Moreover, reduced viscosity exhibited a linear increasing trend with solution concentration ranging from 1.0 to 5.0 mg/mL. Similarly, both intrinsic viscosity (η_i) and average molecular weight (M_w) decreased progressively with increasing hydrolysis time.

These observations align with the findings of previous authors on corn starch. These results could be attributed to the reduction in starch chain length and molecular weight induced by acid hydrolysis; the formation of numerous low-molecular-weight dextrins contributed to the decreased intrinsic viscosity of starch following hydrolysis [6].

3.4. Fourier-transform infrared spectroscopy (FTIR)

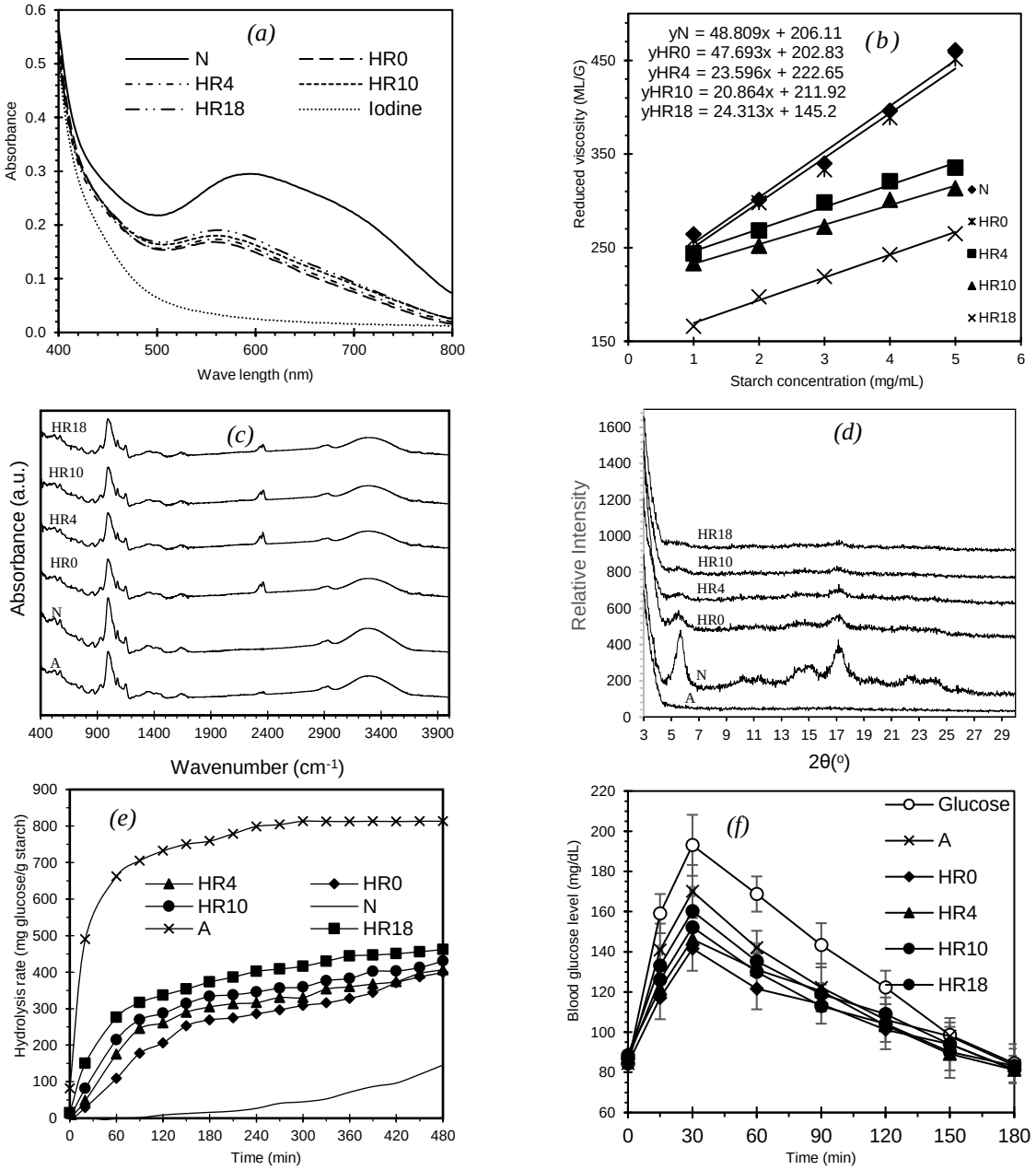

Figure 1. *Some structural characteristics and digestibility of the starch samples; (a) Absorbance; (b) Reduced viscosity; (c) FTIR spectra of starches; (d) XRD spectra of starches; (e) Hydrolysis rate of starches by α-amylase; (f) The formation of blood glucose during the digestion of starch samples.*

The FTIR spectra of the starch samples (Figure 1c) revealed similar main absorption features, indicating that the fundamental chemical structure of the polysaccharide was unchanged by the modifications. However, subtle changes in short-range molecular order were identified, particularly through the absorbance ratio between the band at ~1047 cm^{-1} (associated with the ordered α-helical region) and the band at ~1022 cm^{-1} (associated with the amorphous region), a method used to reflect changes in crystallinity [25].

In this study, the data showed a clear trend: the α-helix/amorphous ratio progressively decreased as the hydrolysis time increased (Table 2). The HR18 sample exhibited the lowest ratio among the treated starches. This indicates that, although the retrogradation stage aims to reconstruct order, the initial shortening of the starch chains by acid treatment diminished their ability to re-align into well-ordered helical structures at the molecular level. This aligns with previous findings where significant degradation, leading to a decrease in molecular weight, was observed through FTIR [26].

This trend is in stark contrast to what is observed in "constructive-refinement" modification methods. Specifically, in the thermal cycling procedure by a previous study [5], this same absorbance ratio systematically increased with the number of processing cycles, signifying an enhancement of molecular order. This opposition provides strong evidence for the fundamental mechanistic difference: (a) in our "destructive-reconstructive" pathway, the initial chain scission permanently limits the potential to re-establish a high degree of molecular order; (b) in the "constructive-refinement" pathway, long, intact chains are "annealed" to achieve a more highly ordered state.

Therefore, the FTIR analysis not only confirms a structural change but also reinforces the central thesis that these two modification strategies produce fundamentally different molecular architectures at the short-range level.

3.5. X-Ray Diffraction (XRD) and Degree of Relative Crystallinity (DRC)

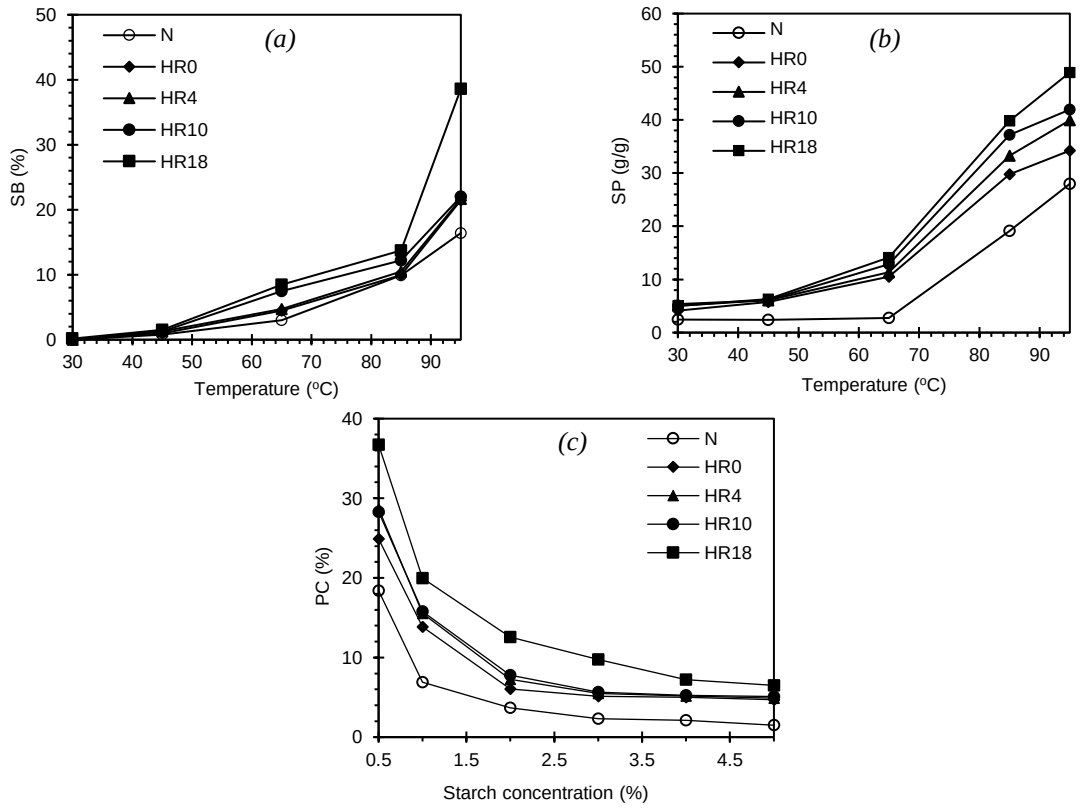

Figure 2. *Physicochemical properties of starchs; (a) Solubility (SB); (b) Swelling power (SP); (c) Paste clarity (PC).*

The X-ray diffraction (XRD) spectra of the starch samples are presented in Figure 1d, with the corresponding degree of relative crystallinity (DRC) values listed in Table 2. Native water yam starch

(N) exhibited a characteristic B-type crystalline structure, displaying major diffraction peaks at approximately 5.7° and 17.1° (2θ), consistent with previous findings [5]. The gelatinized control sample (A) showed an almost completely amorphous pattern, confirming the loss of crystalline order due to heat treatment.

All modified samples (HRs) retained the B-type crystalline pattern; however, the peak intensities and the quantitative DRC data revealed a clear trend: the degree of relative crystallinity systematically decreased as the hydrolysis time increased. The sample HR0, which was retrograded without prior acid hydrolysis, had the highest DRC among the modified samples at 73.85%, while HR18 had the lowest at 53.14%.

This systematic reduction in DRC can be explained by viewing the dual-modification method as a "destructive-reconstructive" process. The initial acid hydrolysis is an intentional "destructive" step that cleaves and shortens the amylose and amylopectin chains, primarily in the amorphous regions [6]. The subsequent retrogradation is a "reconstructive" attempt to rebuild crystalline order from these shorter fragments. However, these shorter chains, despite being more mobile, cannot pack as efficiently into large, well-ordered crystalline structures as the long, intact chains, leading to a systematic decrease in DRC with more extensive hydrolysis.

Table 2. *Ratio of α–helix (1047cm^{-1})/amorphous (1022cm^{-1}) regions and Degree of relative crystallinity (DRC) of starches.*

Samples	Abs_{1047}	Abs_{1022}	α-helix/amophous	DRC (%)
N	0.38	0.49	0.77^{b}	79.54 ± 1.35^{e}
A	0.33	0.46	0.73^{a}	0.4 ± 0.10^{a}
HR0	0.42	0.52	0.81^{f}	73.85 ± 1.10^{e}
HR4	0.39	0.49	0.80^{e}	70.86 ± 2.07^{d}
HR10	0.37	0.47	0.79^{d}	65.49 ± 1.28^{c}
HR18	0.39	0.50	0.78^{c}	53.14 ± 1.14^{b}

Within each column, statistically significant differences ($p<0.05$) are indicated by different superscript characters.

This outcome stands in stark contrast to purely physical, "constructive" modification methods. For instance, a study on thermal cyclic retrogradation of the same water yam starch found that applying thermal cycles to unhydrolyzed starch increased the DRC from 67.6% (native) to as high as 73.2% after nine cycles [5]. In that process, long, intact chains were refined and perfected into a more ordered state. In the present study, however, the initial destructive step permanently compromises the starch's ability to form long-range crystalline order. This unique architecture - characterized by lower long-range crystalline order but potentially containing dense, imperfect crystalline regions - is the key factor responsible for the altered physicochemical properties and modified glycemic index of the starch.

3.6. Solubility (SB), swelling power (SP) and paste clarity (PC)

Solubility and swelling of starch are physicochemical properties closely related to the ratio and the interaction between the crystalline and amorphous regions of starch. The observed differences in solubility and swelling behavior are the direct functional expression of the distinct molecular architectures created by the two modification strategies.

In this study, both solubility (SB) and swelling power (SP) increased with hydrolysis time (Fig 2a/b). Figure 2a showed that the solubilities of the modified starches were not different at temperatures below 45°C but higher at 65-95°C compared to the native starch. This is because the smaller fragments (lower M_w, Table 1) and the less crystalline structure (lower DRC, Table 2) are more easily disrupted upon heating in water, allowing for greater water uptake (swelling) and dissolution of the small fragments (solubility), which is consistent with literature on acid-hydrolyzed starch [6].

Figure 2b showed that modified starch samples (HRs) exhibited higher swelling capacity compared

to native starch (N). At temperatures above 45°C, the swelling capacity of the modified starch samples was significantly higher than that of the native starch (N) and increased with the degree of hydrolysis (HR0<HR4<HR10<HR18). The swelling capacity of starch granules refers to their ability to retain water, which is a characteristic of amylopectin. In contrast, amylose is considered an inhibitor of swelling. The results in Table 1 showed that the amylose content of sample N was the highest, leading to the lowest swelling capacity at all temperatures. It could be observed that the α-helix/amorphous ratio and DRC of the HRs samples were inversely proportional to their swelling capacity.

In contrast, in the study by a privious study [5], both solubility and swelling power decreased with more retrogradation cycles at temperatures above 65°C. This was a result of the increased DRC and the formation of a more robust network from long, intact chains, which restricted water penetration and the leaching of starch molecules.

Figure 2c shows the paste clarity (PC) of native and modified starch samples after gelatinization at 95°C for 30 min. For all starches, PC decreased with higher starch concentration. At the same concentration, PC of the modified starches was higher than that of the native sample (N) and increased with the degree of hydrolysis. According to a previous report, acid-hydrolyzed starch has been reduced molecular weight, resulting in increased clarity of the starch suspension. The results of this study were similar, with sample HR18 having the smallest molecular weight (Table 1) and the highest clarity among the modified starch samples.

3.7. Hydrolysis rate

The research results have shown that hydrolysis rates of the starch samples were arranged in the following order: N<HR0<HR4<HR10<HR18<A (Figure 1e). The starch sample A was completely gelatinized into an amorphous form, making it easier to be attacked by the amylase [27]. Conversely, native starch (N) existed in a granular form with a semicrystalline structure, which provided greater resistance to amylase hydrolysis, resulting in the lowest hydrolysis rate [28]. For the modified starches (HRs), the rate of hydrolysis by amylase increased with the duration of acid treatment due to the decreased DRC (Table 2).

Previous studies have shown that type A crystalline starch is hydrolyzed much faster than types B and C crystalline starch [28]. The high hydrolysis rate of type A crystals is attributed to the high proportion of short amylopectin branches (degree of polymerization 6-12) and the porous structure of the granules [27]. Type B starch exhibits greater resistance to acid hydrolysis compared to type A. The high density of carbohydrates in the amorphous regions, due to the longer amylopectin chains in type B crystals, contributes to higher acid resistance [6]. Therefore, it can be suggested that the type B crystalline structure of water yam starch contributes to the lower hydrolysis rate of the starch samples.

3.8. In vitro glycemic index (eGI) và in vivo glycemic index (iGI)

Table 3. *eGI and iGi of starches.*

Sample	k (1/min)	$C = C_\infty (1 - e^{-kt})$	eGI (%)	iGI (%)
N	n/a	n/a	6.76^{f}	38.69^{f}
A	0.04	788.20	100.00^{a}	72.63^{a}
HR0	0.01	434.94	35.25^{e}	51.07^{e}
HR4	0.01	395.79	39.64^{d}	57.24^{d}
HR10	0.01	377.44	43.28^{c}	61.11^{c}
HR18	0.01	396.23	49.43^{b}	64.67^{b}

Within each column, statistically significant differences ($p<0.05$) are indicated by different superscript characters.

The results of measuring eGI and iGI of starch samples were described in Table 3. Both GI indices were inversely proportional to the ratio of alpha helix/amorphous structures and DRC of the starch samples. An increase in the crystalline structure ratio enhanced structural stability, making them more

resistant to hydrolytic enzymes and reducing glycemic indices. Additionally, for hydrolyzed starch samples, longer acid hydrolysis times resulted in higher eGI and iGI values for modified starch. Starch hydrolysis reduced the molecular size of amylose/amylopectin segments, making it difficult for them to reestablish crystalline structures during the retrogradation.

The discrepancy between the *in vitro* estimated glycemic index (eGI) and the *in vivo* glycemic index (iGI) is not a methodological flaw, but a significant scientific finding in itself. Table 3 shows that all eGI values (35.25% - 49.43%) were approximately 30% lower than the iGI values (51.07%-64.67%). This finding highlights the inherent limitations of simplified *in vitro* systems and reinforces the superior value of *in vivo* results.

This discrepancy can be explained by the complexity of a biological system. The *in vivo* environment in the mouse involves mechanical processing (chewing in the oral cavity, stomach contractions, and movement of the small intestine), chemical factors (a much more complete suite of digestive enzymes, acidic environment in the stomach, and alkaline environment in the small intestine), pH changes, and particularly the physiological activities of the living organism such as hormonal feedback, all of which are absent in the static, single-enzyme *in vitro* model [7]. The higher iGI values suggest this complex *in vivo* environment is more effective at breaking down these modified starches than the simple model predicted.

This finding has a crucial implication: low eGI values reported in studies relying solely on *in vitro* methods, such as that by a previous study [5], may not fully translate to the expected low glycemic response in a living system. Our results demonstrate that *in vivo* GI values should be considered the ultimate measure of their functional performance.

4. Conclusions

This work elucidates a novel "destructive-reconstructive" pathway for engineering starch functionality. Our findings reveal a critical trade-off: while acid hydrolysis creates mobile chains for retrogradation, it also reduces the chain length necessary to form a maximally enzyme-resistant network. Hydrolysis time, therefore, acts as a precise control parameter to tune the final glycemic index. More fundamentally, by showing the significant discrepancy between in vitro and in vivo results, this study underscores the indispensable role of physiological validation in functional carbohydrate development. This provides a promising foundation for producing low-GI food products to address needs related to overweight, obesity, and diabetes.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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