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Effect of Varying Degree of Substitution on Functional and Structural Properties of Kodo Millet Starch

DEEP SHIKHA, SUKHCHARN SINGH and D.C. SAXENA*,

Department of Food Engineering & Technology, Sant Longowal Institute of Engineering & Technology, Longowal-148106, India

*Corresponding author: E-mail: dcsaxena@yahoo.com

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The functional and structural properties of kodo millet starch were investigated as a function of the degree of substitution during esterification with 2-octenyl-succinic anhydride (OSA) at three concentrations (0%, 1.0%, 2.0% and 3.0%). The increasing degree of substitution (0.0058 to 0.0077) indicates preferential substitution of OS groups in the amorphous region of starch. X-ray diffraction revealed a slight decrease in relative crystallinity (31.37-29.87%), indicating that esterification induced structural modifications in both amorphous and crystalline domains. Fourier transform infrared spectroscopy detected peaks around 1720 cm^{-1} and 1645 cm^{-1}, confirming OS group substitution. Microscopic analysis revealed particles with increased surface roughness, indicating that esterification occurs at the surface. The amphiphilic nature of OSA contributes to improved oil-binding capacity, swelling and solubility at increasing temperatures (60 ºC, 75 ºC and 90 ºC). However, water-binding capacity did not change significantly. Pasting properties showed an increase in peak viscosity reflecting improved granule swelling behaviour. The shear-thinning and viscoelastic behaviour observed through rheological assessments indicates that esterification effectively improves the functional performance of kodo millet starch, making it a promising ingredient for diverse food formulation applications.

Keywords: Kodo millet starch, 2-Octenyl succinic anhydride, Esterification, Degree of substitution.

INTRODUCTION

Starch, a primary plant reserve and one of the most abundant biomaterials, is known for its diverse applications including biocompatibility, renewability, low extraction cost and ease of modification. Its versatility, due to its unique film-forming, functional, nutritional and biodegradable properties, makes it valuable across various industrial sectors. Research continues to explore its potential for more sustainable and functional applications [1]. The extraction of starch for the production of starch-based products from major cereal grains and tubers has shifted to other sources due to their lower availability [2]. Millet starches are also now attracting interest due to their health-promoting properties. Kodo millet (*Paspalum scrobiculatum*), a non-conventional starch source categorised as one of the minor millets, is drought-resistant and often termed an orphan or underutilised crop [3]. It belongs to the *Poaceae* family and is mainly cultivated in Vietnam, Nepal, India and Indonesia. Kodo millet grain has a good nutritional profile, comprising carbohydrates (57-60%), proteins (11%), fibre (14.3%), fat (4.2%) and minerals (iron, calcium and potassium), along with essential amino acids [4] and its 65% starch yield comprises about 0.65% minerals, 0.78% fat content and 0.95% protein content using 0.5% alkali extraction [5].

However, native starches have some limitations, such as the strong hydrophilic nature of starch, which makes it less suitable for its application in oil-based products, which can be amended by physical, chemical or enzymatic treatments, either by reprocessing or by modification to widen their applications [4]. The chemical treatment, among other methods, is the most prevalent and traditional method for modifying the properties of starch and is usually carried out with food-grade chemicals in recommended amounts [6]. This involves substituting functional groups, replacing hydroxyl groups in the starch granule, which alters starch characteristics. The chemical modification methods primarily include four types *viz*. acid hydrolysis, etherification, esterification and oxidation [7].

2-Octenyl-succinic anhydride (OSA) esterification of starch is a type of chemical modification that enhances the functional properties of native starch [8]. The modification process involves introducing bifunctional groups to impart amphiphilic properties to starch [9], making it suitable for

applications as an emulsifier, stabiliser and encapsulating agent. The primary characteristic to analyze the efficiency of OSA modification is its degree of substitution (DS). The substitution involves replacing the average number of hydroxyl groups present on starch granules with OSA groups, which depends on the reaction time, conditions, pH range and structural organisation of the granules [10]. Several studies have been conducted on various starch sources, including glutinous rice starch, indica rice starch, japonica rice starch, buckwheat starch, Guinea grass seed starch and Sago starch [11-14] as well as kodo millet starch, but only at single concentrations of OSA (3%) and with dual modifications with other treatments [4,9], to modify their properties. However, the effect of using lower to higher concentrations of kodo millet starch remains unclear. Therefore, the current study aimed to examine the effect of OSA-esterification at varying concentrations on the physico-chemical properties of kodo millet starch.

EXPERIMENTAL

Kodo millet grains were procured from the seed shop in the local market of Sunam city, Punjab state, India and stored under ambient conditions. 2-Octen-1-ylsuccinic anhydride (mixture of *cis*- and *trans*-, 97%, *m.w.*: 210.27 g/mol) was procured from Sigma-Aldrich Pvt. Ltd., India. All other reagents, including sodium hydroxide, hydrochloric acid and phenolphthalein indicator, were of analytical grade and employed in the experimental procedures without further purification.

Starch isolation: Starch was isolated according to the method described by Bist *et al*. [12]. The process starts with washing of kodo millet (500 g) grains and soaking in a 0.25% NaOH solution for 18 h to facilitate the separation of protein and starch. The soaked grains were washed with distilled water three to four times to remove traces of alkali, then wet ground into a fine slurry using a high-speed grinder at 20000 rpm until the granules were converted into a fine slurry. The slurry was filtered through single- and double-layer muslin cloth, then through a 100-mesh sieve. The filtered slurry was kept undisturbed for 4-6 h at a refrigeration temperature (5 ºC) to allow the starch to settle completely at the bottom of the container and the upper layer was discarded. Finally, the slurry was centrifuged at 3000 × g for 15 min using a centrifuge. The supernatant was poured off and the yellow protein layer was scraped off from the top of the extracted starch. The final white starch was dried at 40 ºC for 24 h in a hot air oven and then passed through 100-mesh and 270-mesh sieves. It was stored in airtight pouches. The proximate analysis, conducted using AOAC, 2005 [15] methods, revealed the following results: a moisture content of 10.28 ± 0.25 %, a protein content of 1.88 ± 0.38 %, a fat content of 1.44 ± 0.15 %, an ash content of 0.99 ± 0.04 % and a carbohydrate content of 85.18 ± 0.48 %.

Esterification of starch: Isolated kodo millet starch was esterified using octenyl succinic anhydride, as described by Mahanure *et al.* [4]. A 50 g amount of starch was weighed and suspended in 200 mL of distilled water. Three OSA solutions at concentrations of 1.0%, 2.0% and 3.0% were prepared in acetone. These solutions were slowly added to the starch suspension over 30 min and the pH was maintained at 8.2-8.4 throughout and after the reaction using a 0.5 N alkali solution [11]. After adding OSA, the suspension was incubated for 2 h at 35 ºC with continuous stirring using a magnetic stirrer and then centrifuged at 1928 × g for 10 min using a centrifuge. The sediment was washed with distilled water to remove excess alkali and centrifuged. The starch was dried at 40 ºC for 24 h in a hot air oven. After drying, the ground starch was then passed through a 100-mesh sieve and stored in airtight pouches.

Degree of substitution (DS): The DS was determined using the method described by Chiu *et al*. [17], which involves alkali saponification followed by back-titration of the excess alkali. The DS and reaction efficiency (RE, %), values were calculated using eqns. 1 and 2:

$$DS = \frac{\text{Molecular weight of glucose unit} \times \%\text{OSA substitution}}{100 \times \text{MOSA} - ((\text{MOSA} - \text{MH}) \times \%\text{ substitution}} \quad (1)$$

where molecular weight of glucose residue (162); M_{OSA} = molecular weight of octenyl succinyl group (210); M_H = molecular weight of hydrogen atom (1).

$$RE = \frac{\text{Actual DS}}{\text{Theoretical DS}} \times 100 \quad (2)$$

Theoretical DS was calculated by supposing all added OSA reacted with the starch to form an ester derivative (0.232).

Paste clarity and colour: The paste clarity of 1% starch suspension was determined by measuring its light transmittance (%). A 1% (w/v) aqueous starch dispersion of the starch samples was prepared and heated in a water bath at 95 ºC for 30 min, with continuous stirring at fixed intervals. Then, the cooked paste was cooled at room temperature and its transmittance was measured at 650 nm using a UV-visible spectrophotometer (DR-6000, HACH, USA).

The colour analysis of starch samples was performed using a Hunter Colourimeter equipped with an Optical Sensor, Model D25, with a Quartz Halogen Cycle Lamp illuminant, according to the protocol for calculating L*, a* and b* by the International Commission on Illumination (CIE). Where L* = lightness, +a* = redness, a* = greenness, +b* = yellowness and -b* = blueness.

Amylose content: The amylose content of the starch sample was determined using the method described by Shweta *et al.* [18]. A 0.1 g of starch sample was combined with 1 mL of 95% ethanol and 9 mL of 1 N NaOH, then heated in a water bath at 90 ºC for 10 min. After cooling, it was brought to a total volume of 100 mL with distilled water. A 5 mL portion of this solution was transferred to a 100 mL volumetric flask, followed by the addition of 1 mL of acetic acid and 2 mL of I-KI solution. The mixture was shaken thoroughly and left to stand for 20 min before measuring transmittance at 620 nm with a spectrophotometer (DR-6000 HACH, USA). For the standard, 40 mg of amylose was dissolved in 1 mL of 95% ethanol and 9 mL of 1N NaOH, then heated at 90 ºC for 10 min and marked up to 100 mL with distilled water and labelled as 0% (blank), 4%, 8%, 16% and 20% amylose were used to generate a standard curve from their absorbance readings.

Functional properties

Water absorption capacity (WAC) and oil absorption capacity (OAC): The absorption capacities were determined

at room temperature (30 ± 2 ºC) using the method of Kumar *et al.* [19]. A 1% starch suspension in distilled water and a refined soybean oil suspension were separately prepared for the analysis of WAC and OAC, respectively. The suspension was centrifuged at 3000 ×g for 10 min in a centrifuge. The surplus water and oil were decanted and the tubes were inclined at 45º for 20 min before weighing. The absorption capacities were calculated by using (eqns. 3 and 4).

$$\text{WAC (g/g)} = \frac{(A - B) - W \text{ of sample}}{W \text{ of sample}} \quad (3)$$

$$\text{OAC (g/g)} = \frac{(A - B) - W \text{ of sample}}{W \text{ of sample}} \quad (4)$$

where A = W of tube and sample after removal of oil; B = W of empty tube.

Swelling power and solubility: The swelling power and solubility were determined using a method described by Sharma *et al.* [20] at 60 ºC, 75 ºC and 90 ºC. A 1% starch suspension was placed in centrifuge tubes and vortexed for 10 sec. The samples were heated in a water bath at 60, 75 and 90 ºC for 30 min each, then cooled to room temperature. The samples were then centrifuged at 1928 ×g for 15 min in a centrifuge. The supernatant solubility was measured after each centrifugation cycle. All measurements were done in triplicate.

Rheological properties

Pasting properties: The Rapid Visco-Analyser (RVA, Tech Master Perten Instruments, Sweden) was used to analyse the pasting properties of starch according to the method described by Mir *et al.* [21]. A 3 g starch sample (on a 14% moisture basis) was mixed with 26.04 mL of distilled water, as calculated based on the given moisture content of 9.15%, in a canister and stirred at 960 rpm for a few seconds to ensure proper mixing. After mixing, the suspension was held at 50 ºC for 1 min to preheat the sample, followed by continuous stirring at 160 rpm during cooking. Thereafter, the sample was heated for 3 min and 42 s at a constant heating rate of up to 95 ºC. At 95 ºC, the sample was held for 2.5 min, then cooled to 50 ºC in 3 min 48 sec at a constant cooling rate.

Steady and dynamic properties: A cooked starch paste was obtained from the RVA analysis and cooled at room temperature for 30 min. The steady and dynamic characteristics of the starch gel were determined using a rheometer (MCR 102, Anton Paar, Austria) using a parallel plate system (5 cm diameter) at a measurement gap of 1000 μm.

Structural and morphological characteristics: The morphological characteristics of starch were analysed using a scanning electron microscope (JSM 6610-LV, JEOL, Japan, operating at 5 kV) at a magnification of 5000×, following the method described by Mir *et al.* [21].

The FTIR spectra of native and esterified starches were recorded on an FTIR spectrophotometer (TENSOR 27, Bruker Optics Inc., USA) linked with an ATR plate within the range of 4000 to 400 cm^{-1} at 20-25 ºC.

The X-ray diffraction intensity of native and esterified starch was measured using an X-ray diffractometer (D8 Advance, Bruker, Germany). The scanning range was 5º-60º at 4º/min, with a target voltage of 40 kV and a current of 40 mA.

$$\text{Degree of crystallinity} = \frac{\text{Area of crystalline region}}{\text{Total area under curve}} \times 100 \quad (5)$$

Thermal properties: A differential scanning calorimeter (NETZSCH, Germany) was used to determine starch gelatinisation properties. In brief, 5 mg of the sample was loaded into an aluminum pan and distilled water was added to obtain a 70% water suspension. The aluminum pan was hermetically sealed and equilibrated at room temperature for 1 h. The thermal analysis was performed at a heating rate of 5 ºC/min over the temperature range of 30 to 100 ºC. The onset temperature (T_o), peak temperature (T_p) and conclusion temperature (T_c) were calculated.

Preparation of emulsion: The oil-in-water emulsions of native and modified starch samples were prepared using the method discussed by Jain *et al.* [16]. A 3% (w/v) suspension of native and modified starch was prepared. Further, 90 mL of starch suspension was mixed with 10 mL of sunflower oil using a high-speed homogenizer (MT-30 K, Moxcare, India) at 8000 rpm for 10 min. Subsequently, a high-pressure homogenizer (NS1001 L2K, Italy) was used for further homogenisation. The high-pressure homogenisation process was performed three times at 80 bars. Lastly, sodium azide (0.02 %) was added to the emulsion to prevent microbial growth for subsequent analysis.

Emulsifying index: The emulsifying index of the prepared emulsions was determined using the method discussed by Wang *et al.* [11].

Statistical analysis: All the experiments were performed in triplicate and data were reported as mean ± standard deviation. Analysis of variance at $p < 0.05$ in IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY) was used to assess differences between means.

RESULTS AND DISCUSSION

Degree of substitution (DS) and starch yield: The degree of substitution of OSA-modified kodo millet was significantly increased (0.0058-0.0077), as shown in Table-1. The lower DS values for kodo millet starch were consistent with those of native proso starch [22] which was further enhanced by ultrasonication, suggesting that millet starches showed limited accessibility for ester group substitution in their native state. The degree of substitution in OSA-esterification of starch is influenced by various factors, including reaction time, pH and the composition and structural arrangement of the starch [9]. Sharma *et al.* [20] reported that increasing reaction time significantly improves the substitution of ester groups on the starch backbone of pearl millet starch. The lower DS values of Kodo millet starch indicate an intact structural arrangement, a more crystalline structure and limited accessibility of the hydroxyl groups (C2, C3 and C6) of the starch backbone. The secondary hydroxyl groups (C2 and C3) showed lower or no reactivity toward bulky groups and the steric hindrance of bulky OSA groups further restricts substitution at a higher concentration of OSA (3%), while the probability of interaction between ester and hydroxyl groups is likely to be enhanced. The lower accessibility of hydroxyl groups and their steric hindrance also reduce reaction efficiency (25.00 to 33.18%), indicating that only one-third of the OSA reagent

TABLE-1
EFFECT OF VARYING DEGREES OF SUBSTITUTION ON STARCH YIELD, AMYLOSE CONTENT AND COLOUR

Sample	DS	RE (%)	Starch yield (%)	Amylose content	Paste clarity (%)	Colour values		
						L*	a*	b*
KSOS-0	–	–	–	30.37±0.12^{d}	7.82±0.12^{a}	91.10±0.10^{a}	1.3±0.07^{d}	2.42±0.09^{d}
KSOS-1	0.0058±0.003^{a}	25.00±0.02^{a}	96.46±0.11^{c}	29.47±0.16^{c}	8.57±0.06^{b}	92.89±0.09^{b}	1.02± 0.04^{c}	2.18±0.06^{c}
KSOS- 2	0.0068±0.002^{b}	28.44±0.04^{b}	91.15±0.10^{b}	28.62±0.15^{b}	10.14±0.10^{c}	94.70±0.13^{c}	0.60±0.11^{b}	1.94±0.07^{b}
KSOS-3	0.0077±0.002^{c}	33.18±0.05^{c}	86.36±0.08^{a}	26.98±0.08^{a}	11.47±0.11^{d}	95.31±0.16^{d}	0.48±0.06^{a}	1.67±0.07^{a}

Values are presented as the mean±SD of three replicates, with different superscript letters in the columns indicating a significant difference ($p < 0.05$); KSOS-0 to KSOS-3 represent native kodo millet starch and esterified starches at three different concentrations (1.0%, 2.0% and 3.0%).

reacts with starch and the remaining portion is either hydrolysed under aqueous alkaline conditions or remains unreactive [4,22]. The results revealed that the tightly packed structure of small granules requires physical or enzymatic pretreatment to improve these substitutions.

A comparative analysis of the degree of substitution for minor millet, major millet and cereal sources indicates that the OSA esterification reaction varies with factors such as starch source, accessibility of hydroxyl groups, reaction time, source variety and pre-treatments are shown in Table-2. The starch yield after esterification was evaluated to analyse the effect of increasing the degree of substitution. An increase in OSA concentration significantly ($p < 0.05$) reduces starch yield from 96.46% to 86.36%. The use of alkali during the esterification process may promote greater solubilisation of starch granules, which, in turn, can lead to partial gelatinisation. It may occur during succinylation of the hydroxyl and carbonyl groups in OSA [25].

Paste clarity and colour: The paste clarity (% transmittance) and the colour values of native and treated starches are listed in Table-1. The varying substitution of OS groups in the esterified starches also improves the clarity of the starch paste. Possible reasons include the introduction of carboxyl groups, which form hydrogen bonds with starch granules and the substitution of OS groups in the structural arrangement of starch granules, which inhibits retrogradation and results in a clear fluid [26]. The decreasing positive values for a* signify that the sample appears reddish and lower values indicate greater clarity and less brown pigmented modified starch. The increased b* value indicated that the native starch contained proteins and lipids, whereas the modified starch had fewer [27]. Starch is white in its native state and esterification of starch with OSA significantly improved its brightness, an important parameter from an industrial perspective for gel-based food products such as jam, jellies and fruit paste.

Amylose content: Table-1 presents the amylose content of native and OSA-modified starch. The reduction in amylose content of esterified starch may be attributed to similar steric hindrance caused by attached groups on hydroxyl groups, which interfere with iodine binding. One possible reason for the decreasing values is that esterification of starch adds bulky ester groups, which may interact with the amylose chain and hinder amylose leaching [11]. Another possible reason is the structural complexity introduced by the esterification process, which primarily disrupts the amorphous regions, potentially interfering with iodine absorption and reducing amylose detection [13]. The negative correlation between amylose and the degree of substitution of KSOSs was similar to that observed in potato and japonica rice starch, with an increasing degree of substitution [28,29].

Functional properties

Water and oil absorption capacity: Table-3 shows the water absorption capacity of treated and untreated KSOSs. The water-binding capacity of starch is influenced by various parameters, including the presence of free -OH groups and the type and intensity of substitution groups [30]. The change in the values was likely due to a reduced availability of free hydroxyl groups in esterified starch, either because bulky ester groups or long hydrophobic alkyl chains were added.

The structural integrity and molecular rigidity of OSA-modified starch may remain intact, thereby reducing granular water absorption. The results were consistent with studies reported by Singh *et al.* [27] for Chenopodium starch under different pH conditions. The increase in water-binding capacity at lower concentrations might be due to the introduction of

TABLE-2
COMPARATIVE ANALYSES FOR THE DEGREE OF SUBSTITUTION WITH DIFFERENT STARCH SOURCES

Starch Source	OSA conc. (%)	DS	Observations	Ref.
Kodo millet	3	0.0067	Lower DS in native starch	[4]
Kodo millet	3	0.0066	Similar results	[9]
Kodo millet (cold plasma; 10-30 kV, 5-10 min)	3	0.0077-0.0274	Cold plasma treatment significantly improves the DS value	[23]
Proso millet (ultrasonication, 35%, 10/20 min)	3	0.0078-0.0115	Minor millet starch, DS improved after ultrasonication, but is still lower than other sources	[22]
Pearl millet (reaction time, 2-5 h)	3	0.018-0.022	Major millet, improved results with reaction time	[20]
Buckwheat	0.6-3	0.0032–0.0229	Increases with increased concentration	[11]
Rice indica	3	0.0287	High DS value, more emulsifying property	[24]
Varieties of rice	3	0.0093-0.0159	Varied with rice varieties	[10]

TABLE-3
EFFECT OF VARYING DEGREES OF SUBSTITUTION ON ADSORPTION CAPACITY, SWELLING POWER AND SOLUBILITY OF KODO MILLET STARCH

Sample	WAC (g/g)	OAC (g/g)	SP (g/g)			WSI (%)		
			60 ºC	75 ºC	90 ºC	60 ºC	75 ºC	90 ºC
KSOS-0	1.87±0.09^{a}	2.55±0.09^{a}	3.50±0.02^{a}	7.09±0.06^{a}	10.28±0.18^{a}	15.42±0.14^{a}	19.18±0.10^{a}	35.09±0.04^{a}
KSOS-1	1.96±0.09^{b}	2.62±0.03^{b}	3.58±0.10^{ab}	7.58±0.04^{b}	11.46±0.08^{b}	15.97±0.11^{b}	20.15±0.18^{b}	35.89±0.09^{b}
KSOS-2	1.89±0.09^{a}	2.78±0.02^{c}	3.62±0.05^{b}	8.38±0.12^{c}	12.68±0.11^{c}	16.32±0.04^{c}	20.98±0.18^{c}	36.44±0.12^{c}
KSOS-3	1.88±0.09^{a}	2.90±0.08^{d}	3.68±0.07^{bc}	8.80±0.09^{d}	13.49±0.05^{d}	17.31±0.07^{d}	21.54±0.18^{d}	37.03±0.07^{d}

Values are in mean±SD of three replicates, with different superscript letters in the columns indicating a significant difference ($p < 0.05$). KSOS-0 to KSOS-3 represent native kodo millet starch and esterified starches at three different concentrations (1.0%, 2.0% and 3.0%).

more hydrophilic groups or the reduced capability of the hydrophobic chain to replace hydroxyl groups in starch granules. The decrease in water-binding capacity with increasing OSA concentration reflects the presence of more hydrophobic groups replacing the hydroxyl groups on the starch chain.

The oil-binding capacities of native and esterified starch are shown in Table-1. The property defines the presence of hydrophobic sites available on the molecular chains and surface morphology of starch. The oil-absorption capacity of esterified starch increased with concentration, exhibiting a higher oil-binding capacity than native starch, in agreement with previous studies on pearl millet starch [20]. The improvement in the oil-absorption properties of esterified starch is closely associated with surface roughness and the substitution of ester groups on the available sites of starch molecules [30]. Introducing hydrophobic alkyl chains and increasing the degree of substitution improve the affinity of OAC [20].

Swelling capacity and solubility: The capability of starch granules to swell (absorb water and form a gel-like consistency) upon heating, cooling and centrifugation is known as swelling capacity (Table-3). The swelling power of OSA-treated starches increased substantially with increasing temperature, from 3.50 g/g to 13.49 g/g. Higher values of swelling power at higher temperatures were found to be a function of temperature, due to weakening of hydrogen bonds between hydroxyl groups and the penetration of more water into the starch granules [31]. The values did not change significantly at lower temperatures, possibly because amylose leached from starch in limited amounts [12]. Similar findings were reported for waxy maize and amaranth starches [32].

As the solubility of starch is an important parameter, the solubility of kodo millet starch varied significantly ($p < 0.05$) with increasing OSA concentrations (1.0-3.0%) as a function of degree of substitution and increased with increasing temperature (60, 75 and 90 ºC). The solubility of starch improved with the substitution of OSA groups in the amorphous region of starch, simultaneously facilitating structural reorganisation and disruption of hydrogen bonds, thereby enhancing the interaction of hydroxyl groups with free water molecules near the starch granules and improving starch solubility [20].

Rheological properties

Pasting properties: The pasting properties of starch include heating (gelatinisation) and cooling (retrogradation) cycles (Table-4 and Fig. 1). Starch granules swell upon heating above the gelatinisation temperature, indicating the peak viscosity and the initiation of this process indicates the pasting temperature. The native kodo millet starch exhibited a higher pasting temperature (84.70 ºC) and a lower peak viscosity (1947 cP), indicating a more ordered and intact molecular structure and greater resistance to swelling. At the same time, a high peak viscosity was observed in esterified starches, which is interpreted as indicating a good water-holding capacity based on the shearing ability of the granules [33].

The ordered structure of native starch might be disrupted by the amalgamation of a long chain of hydrophobic OSA groups and the breakdown of hydrogen bonds, resulting in improved water penetration at lower pasting temperatures [20]. Pasting temperature of esterified starch (83.95-82.30 ºC) was lower than that of native starch (84.70 ºC), confirming that disruption of the intact structure and induced hindrance to weaken hydrogen bonds occurred over esterification [20]. The breakdown value (BDV) describes the loss in viscosity under high shear and the disintegration of swollen granules at high temperatures [34]. The continuous application of heat causes the breakdown of all crystalline regions within the granules, thereby reducing viscosity. Higher breakdown values for OSA-treated starches showed higher swelling capacity but lower stability at higher temperatures.

The final viscosity (FV) of esterified starch explains the ability of starch granules to re-associate after cooling, measured at ~50 ºC. The decreased final viscosity of treated starch

TABLE-4
EFFECT OF OSA MODIFICATION ON THE PASTING PROFILE OF KSOSs

Sample	PT (ºC)	PV (cP)	TV (cP)	FV (cP)	BD (cP)	SB (cP)
KSOS-0	84.70 ± 0.07^{c}	1947 ± 15.5^{a}	1165 ± 20.5^{a}	3313 ± 19.0^{c}	782 ± 32.5^{a}	2142 ± 14.8^{d}
KSOS-1	83.95 ± 0.03^{b}	2588 ± 16.9^{b}	1333 ± 17.6^{d}	3307 ± 14.8^{c}	1255 ± 18.3^{b}	1980 ± 15.5^{c}
KSOS-2	82.35 ± 0.03^{a}	2806 ± 12.7^{c}	1205 ± 15.5^{c}	2999 ± 26.8^{b}	1601 ± 16.2^{c}	1794 ± 16.9^{b}
KSOS-3	82.30 ± 0.03^{a}	2881 ± 21.9^{d}	1185 ± 14.8^{b}	2853 ± 12.0^{a}	1696 ± 12.0^{d}	1668 ± 18.6^{a}

Values are in mean ± SD of three replicates, with different superscript letters in the columns indicating a significant difference ($p < 0.05$); where PT- pasting temperature, PV- paste viscosity, TV- trough viscosity, FV-final viscosity, BD- breakdown and SB- setback.

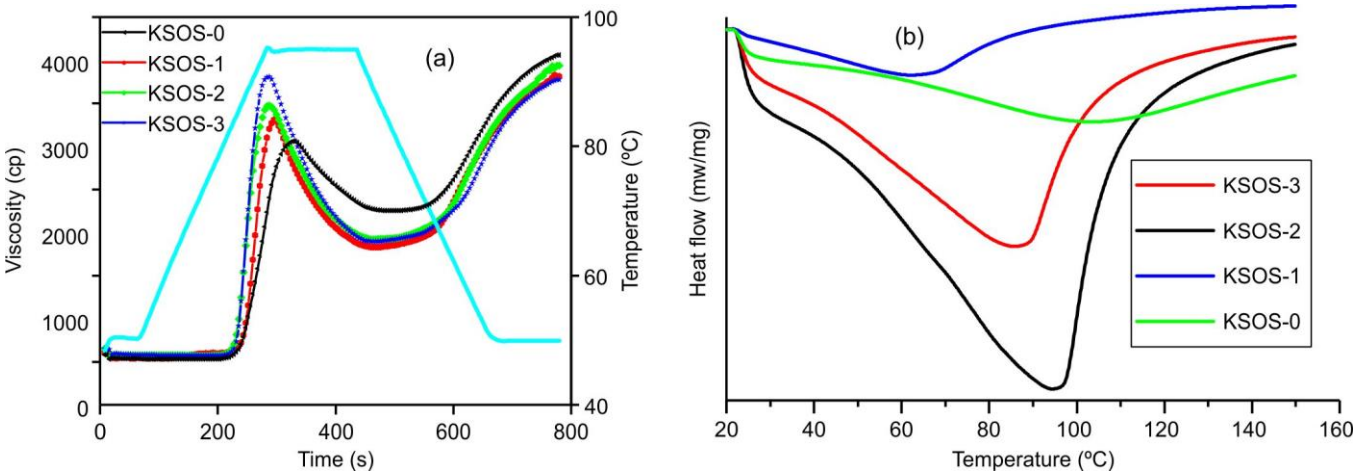

Fig. 1. Effect of OSA esterification on (a) pasting properties and (b) thermal properties of KSOSs

compared to native starch indicates steric hindrance by OSA groups, which limits amylose association and results in a weaker gel structure. The retrogradation phenomenon in starch pastes, which occurs during cooling can be expressed as a setback. Lower setback values indicate lower re-association of starch molecules and a softer paste texture, which is favourable for the preparation of spreads, soups and baby foods. The results were consistent with studies on OSA for waxy maize, rice, wheat B-type, oat, quinoa and Amaranth starch [33]; as well as rice, wheat and potato starch [35].

Steady and dynamic rheological properties: The plots of shear stress (σ) *vs.* shear rate (γ) and viscosity *vs.* shear rate (γ) of native and OSA-modified KSOSs are shown in Fig. 2. In steady shear rheological properties, increasing σ and decreasing viscosity with increasing shear rate (γ), showing shear-thinning (non-Newtonian) flow behaviour for all starch samples. The shear thinning behaviour of starch was prominent for all starch samples, mainly with a higher concentration of OSA (3%). The shear stress increased continuously with the shear rate, possibly due to the structural breakdown of intermolecular bonds in starch granules caused by the incorporation of OSA groups [20]. The maximum viscosity was observed in native kodo millet starch, likely due to intact amylose and amylopectin chains. In contrast, a decrease in viscosity with increasing substitution indicates successful OSA modification. This decline, especially under continuous mechanical shearing, suggests a progressive breakdown of the gel structure formed by amylose molecules [9]. Another possible reason is amylose leaching after gelatinisation, which can also affect the behaviour of starch gel [33].

The dynamic rheology of starch reveals its viscous and elastic nature. The values of G′ and G″ are shown in Fig. 2. The effect of OSA-esterification showed that the magnitudes of storage modulus and loss modulus were increasing similarly to that of native starch. The G′ value was higher than G″, which signifies strong gel formation for all starch samples at higher frequencies. The higher values for the storage modulus were observed for native starch and with modification, the values decreased, possibly due to the breakdown of inter and intra-molecular bonds between amylose and amylopectin, as well as steric hindrance of OSA groups, which limits the interaction between starch granules [9]. The G′ > G″ values, with no crossover found during the experimental analysis, indicate that the viscoelastic nature of all starch gels was prominent [20].

Structural and morphological properties

Morphological properties: Scanning electron microscopy was used to analyse the surface morphology of native and modified starch at 5000× magnification; micrographs are shown in Fig. 3. The micrographs showed that kodo millet starch, without any treatment, has a mixture of oval and poly-

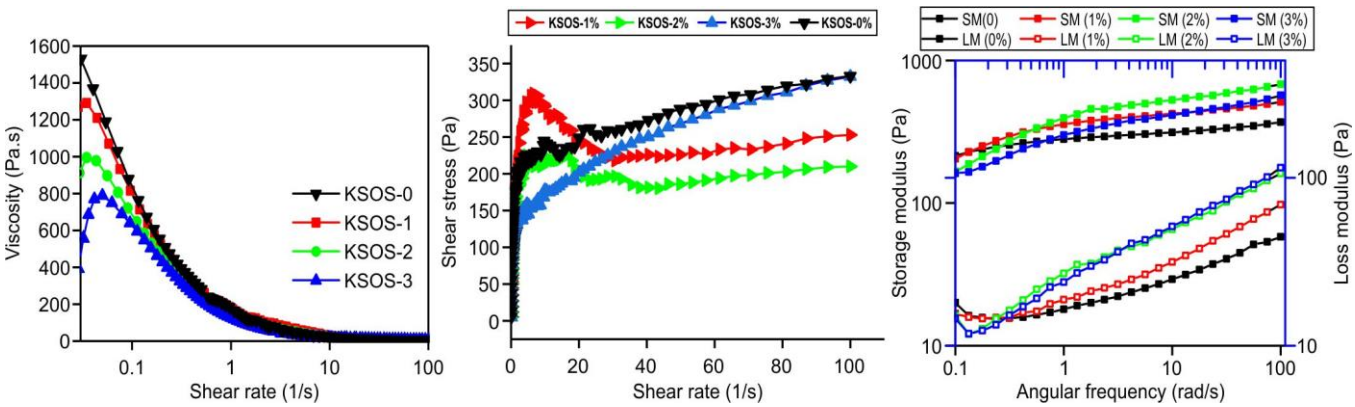

Fig. 2. Effect of OSA modification on steady and dynamic rheological properties of KSOSs esterified at (0%, 1.0%, 2.0% and 3.0%)

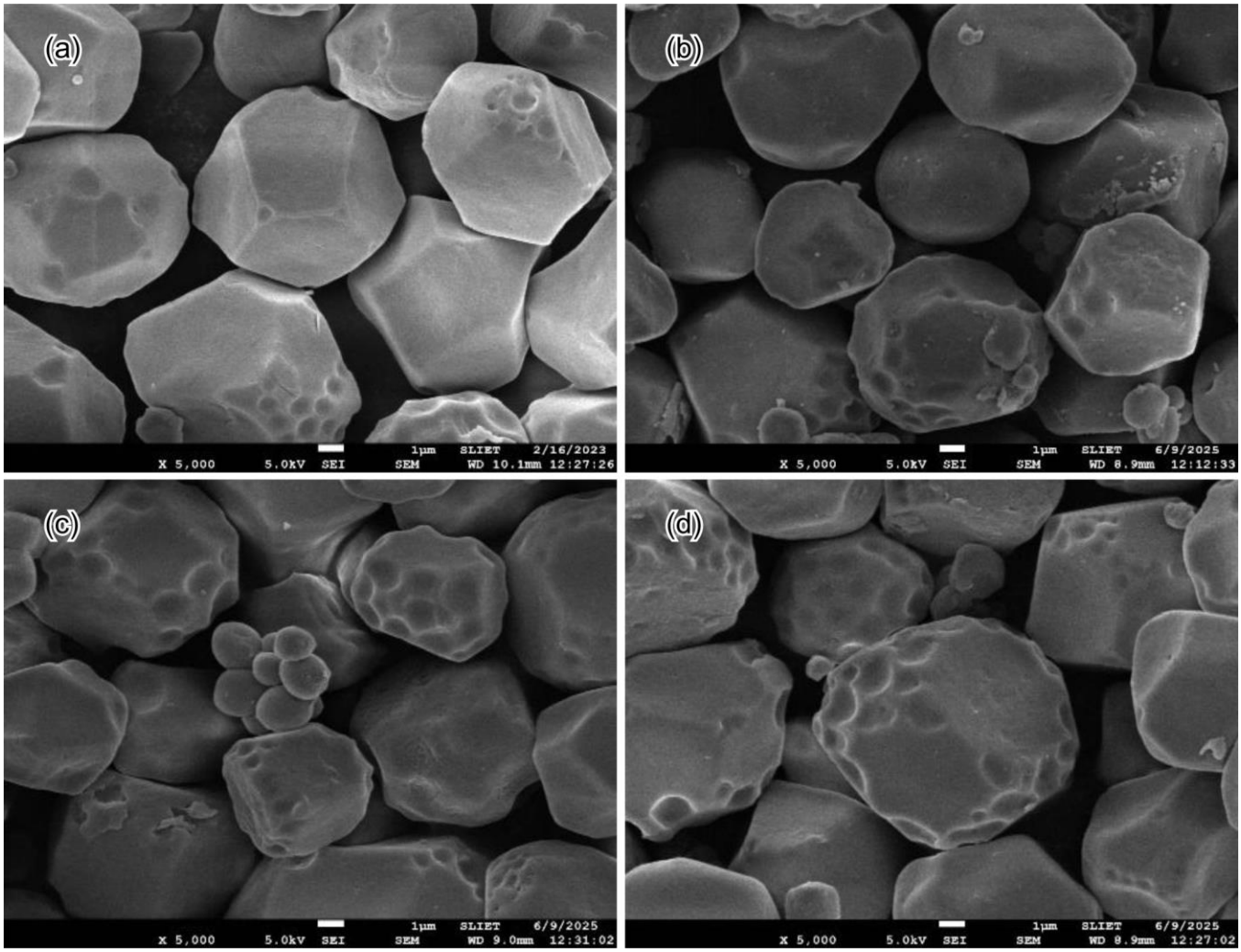

Fig. 3. Effect of OSA modification on morphology of starch: (a-d) OSA (0%; 1.0%; 2.0%; 3.0%) at 5000X

gonal shapes with sharp edges and a smooth texture. On the other hand, upon OSA modification, surface smoothness decreases with increasing substitution degree. The rough edges and irregular granule shape increased the substitution of OSA groups.

Surface disintegration can be attributed to the use of alkali during the esterification reaction, as gelatinisation can occur while maintaining the reaction pH [13,36]. These alterations in the surface morphology of starch were purely found on the edges, without any surface cracks. Similar results were observed for buckwheat starch [11] and sago starch [13]. The increased surface roughness can also be attributed to partial substitution of hydroxyl groups with octenyl hydrophobic groups [9].

X-ray diffraction studies: The diffraction pattern at the 2θ angle and the peak intensities indicated starch crystallinity (A, B and C types) from various sources, including cereals, millet, tubers and roots. The diffraction of kodo millet starch samples with strong peak intensities at 2θ = 15.5º, 17.3º, 18.3º and 23.03º confirmed the A-type crystalline pattern of starch as of native starch (Fig. 4a). During modification with OSA, the changes in peak intensity and area under the crystalline region were analysed to distinguish the changes occurring in modified starch. The relative crystallinity (31.37-29.87%) of the modified starch samples decreased with an increasing degree of substitution [11]. Studies have shown that OSA esterification primarily occurs in the amorphous regions [37], but it may also affect the crystalline pattern to some extent. This could also be attributed to partial melting of the crystalline region or to altering the orientation of the double-helical structure of starch [9].

Additionally, starches obtained from Andean tubers (oca and olluco) exhibit reduced crystallinity following OSA modification [38]. The relative crystallinity of starch depends on several factors *viz*. chemical composition, double helix structural orientation and processing conditions [39,40]. Changes in the area under the crystalline regions may be due to the use of alkali during the modification process, which alters inter- and intra-molecular bonds. The results were consistent with OSA esterification of proso millet starch [22]. Exceptions with no change in relative crystallinity are reported for sorghum starch [37], suggesting that mild alkali conditions during processing may not alter its crystalline regions.

FT-IR analysis: The chemical composition of the control and starch modified with OSA was investigated using FTIR spectra (Fig. 4b). The functional group in the 4000-1500 cm^{-1} region showed a broad absorption band centred at 3400-3200

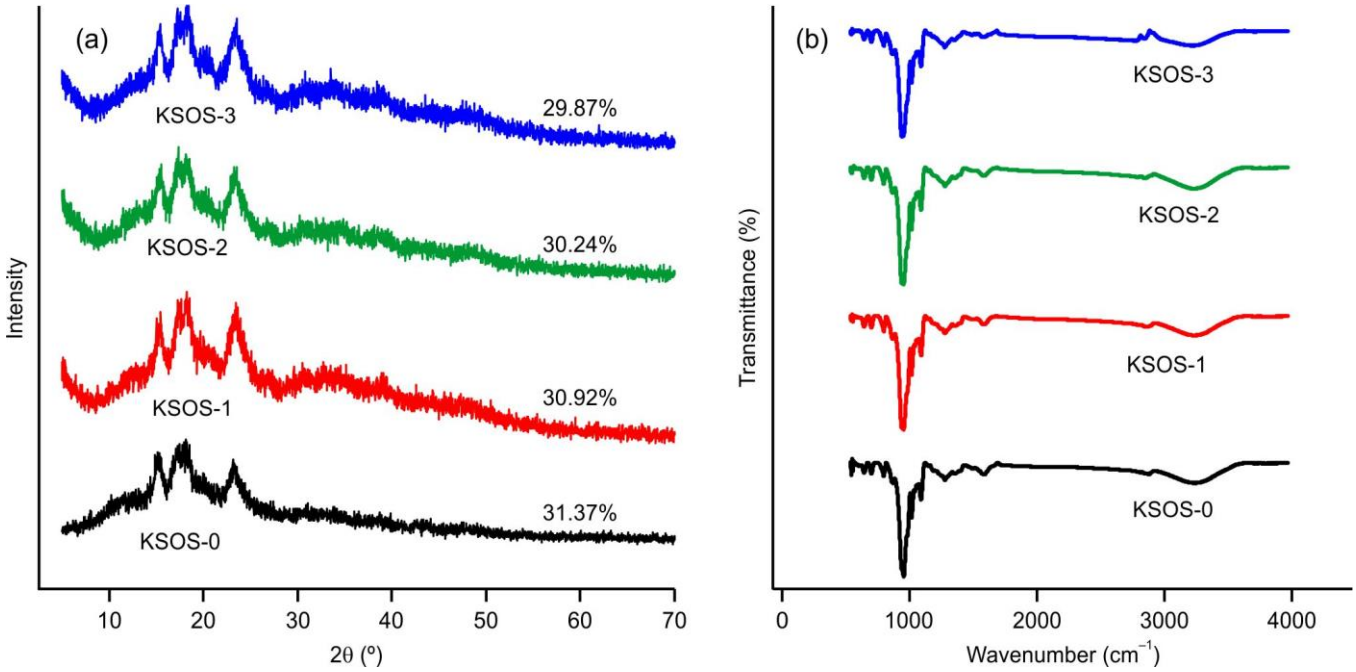

Fig. 4. Effect of OSA modification on (a) X-ray and (b) FTIR spectra of KSOSs

cm^{-1}, attributed to O-H stretching of -OH groups. The increase in peak intensity in this range indicates enhanced water-retention properties of OSA-treated starches [41]. In the fingerprint region (1500-500 cm^{-1}), the absorption spectra in the range of 1300-900 cm^{-1} are attributed to the C-O and C–C vibrational modes. The intensity of bands in this range indicates a degree of starch gelatinisation. The decreasing intensity with modification indicates successful attachment of groups.

In the esterified starch, absorption peaks were observed around 1720 cm^{-1}, corresponding to the ester carbonyl (C=O) stretching vibrations, which confirms successful esterification and peak at 1645 cm^{-1} corresponds to the stretching vibrations of C=O groups in ester carbonyl groups [42]. These new peaks confirm the OS-group substitution and the carbonyl group replaces the hydroxyl group in starch granules. Furthermore, the decreased absorbance ratios (995: 1022 cm^{-1} and 1047: 1022 cm^{-1}) indicate a reduced degree of molecular order, leading to disruption of the crystalline region upon OSA application and confirming the binding of OSA groups (Table-5).

TABLE-5
SHORT-RANGE ORDER AND EMULSIFYING INDEX (EI) VALUES OF CONTROL AND OSA ESTERIFIED KODO MILLET STARCH

Sample	995:1022 cm^{-1}	1047:1022 cm^{-1}	EI (%)
KSOS-0	1.028 ± 0.008^{d}	1.712 ± 0.005^{d}	18.5 ± 0.02^{d}
KSOS-1	1.009 ± 0.002^{c}	1.200 ± 0.006^{c}	29.4 ± 0.04^{c}
KSOS-2	1.000 ± 0.007^{b}	1.149 ± 0.012^{b}	37.2 ± 0.03^{b}
KSOS-3	0.994 ± 0.006^{a}	1.07 ± 0.010^{a}	50.8 ± 0.02^{a}

Values are in mean ± SD of three replicates, with different superscript letters in the columns indicating a significant difference ($p < 0.05$).

Thermal properties: The gelatinisation properties of esterified starch and native starch were analysed and characterised by three temperatures *viz.* T_o (onset), T_g (glass transition/peak temperature) and T_c (conclusion temperature) and ΔH (enthalpy of gelatinisation) (Fig. 1b). Gelatinisation is a temperature- and moisture-dependent transition that strongly influences the thermal characteristics of starch. The onset temperature signifies the initiation of crystalline structure melting, the peak gelatinisation temperature corresponds to the maximum extent of crystalline melting and the conclusion temperature indicates the completion of the gelatinisation process. The enthalpy of gelatinisation reflects the energy required to disrupt the ordered double-helical structures within starch granules [10]. The higher values for onset (88.44 ºC), gelatinisation (93.22 ºC) and conclusion temperature (95.87 ºC), along with high enthalpy (17.14 J/g) of native starch, indicate greater structural integrity and a more crystalline structure. However, the increasing substitution of OS groups, which disrupts the molecular arrangement, facilitates the penetration of water molecules [43], resulting in lower values. The gelatinisation of esterified starches (91.67-90.78 ºC) occurs earlier with low onset (87.15-85.51 ºC) and conclusion temperature (94.32-93.13 ºC) at lower energy levels (14.48-16.57 J/g). The results are in agreement with other OSA esterified starch sources [10,13,33,43]. Although the changes in temperature were minor due to a lower degree of substitution of OS groups as compared to other starch sources, which could be related to minimal changes in degree of crystallinity.

Emulsifying index (EI): The emulsifying index of isolated native and esterified kodo millet starch was analysed by measuring the emulsion height after 24 h at 25 ºC (Table-5). Lower native starch values were associated with a hydrophilic nature and surface-active properties created by larger particles, limiting their emulsification capacity [4]. The higher stability observed with higher OSA concentration indicated the presence of ester groups at hydroxyl positions, leading to the amphiphilic properties of modified starch [20]. Improved emulsi-

fying index of OSA starches indicates greater interface stability, thereby reducing coalescence and phase separation.

Conclusion

2-Octenyl-succinic anhydride (OSA) esterification of kodo millet starch substantially changes its functional properties. The decrease in water absorption capacity (WAC) and amylose content suggests that introducing OSA groups hinders hydration and increases amylose leaching due to alkaline conditions. The morphological studies showed that OSA-esterification is a surface phenomenon, as roughness increases with increasing degree of substitution (DS). Shear-thinning behaviour, accompanied by increased gel elasticity, was confirmed by rheological studies. Meanwhile, XRD patterns showed reduced crystallinity with increasing DS. This signifies that changes occur in both amorphous and crystalline regions. New peaks in FT-IR investigation also confirm the OSA reaction with starch molecules. The thermal properties with the substitution of OS groups revealed a decrease in gelatinisation temperature and energy required for gelatinisation. These findings demonstrate that even a low degree of substitution achieved through esterification can significantly influence the structural and functional characteristics of native starch, thereby broadening its potential applications in food systems.

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CONFLICT OF INTEREST

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

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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