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# Enzymatic degradation of hydroxypropyltrimethylammonium wheat starches

Ali Ayoub [a], Sebastien Gruyer [b], Christophe Bliard [a,*]

[a] *Laboratoire de Pharmacognosie, Faculté de Pharmacie, UMR 6013 CNRS, Bât 18 Europol'Agro, Moulin de la Housse, URCA, 51687 Reims Cedex 2, BP 1039, France (UE)*

[b] *Laboratoire de Microbiologie Industrielle, Faculté de Sciences, 51687 Reims Cedex 2, France (UE)*

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**Abstract**

The enzymatic degradation of hydroxypropyltrimethylammonium modified starches synthesised by dry process was compared with that of hydroxypropyltrimethylammonium modified starches synthesised in glycerol–water plasticised molten medium. The enzymatic degradation rate of products from both origins decreased as the degree of substitution increased. However, two distinct enzymatic degradation profiles were obtained. Dry process products displayed a regular decrease pattern as DS increased. Molten medium synthesised cationic starches displayed a constant degradation level on a wide DS range with α,β-amylase and amyloglucosidase, whereas isoamylase degradation rapidly reached its degradation limit at DSs 0.05. The various plasticising conditions used to synthesise cationic starch in molten medium show no influence on the enzymatic degradation.

By measuring the affinity of α-amylase, β-amylase and isoamylase for native, extruded non-modified and hydroxypropyltrimethylammonium-modified starches. It was evident that the enzymes' affinity for the substrate diminishes with increasing chemical modification, particularly in the case of α-amylase, suggesting that the location of cationic groups impairs the enzyme's recognition of the substrate. Structural elements of limit dextrins were analysed by $^{1}$H NMR.


*Keywords:* Cationic starch; Enzymatic degradation; Kinetics

## 1. Introduction

Chemically modified starches have found numerous applications in food and non-food industries. Functional groups are introduced on the macromolecules by reaction on the hydroxyls groups [1,2]. Cationic starches are widely used in industrial paper-making process [3], where they bind to negatively charged cellulose fibres by ionic association.

Hydroxypropyltrimethylammonium (HPTMA) is one of the most widely used cationic modifying groups. In industrial process, the chemical modification is performed either in solution or by dry process [2]. In a previous paper we described an alternative chemical modification procedure in microhydric molten medium using glycerol-plasticised starch [4].

Hydrolysis has been widely used to study the molecular architecture of complex carbohydrate polymers. Hydrolysis of starch can be performed by acidic treatment [5], or by enzymatic degradation [6,7]. Enzymatic degradation is the base of the largest starch transformation industries. Hamdi and Ponchel [8] have studied the influence and the sensitivity to enzymatic degradation of starch microspheres with α-amylase. Brandam et al. [9] describe the α- and β-amylase activities on starch.

Several research groups have investigated the enzymatic degradation of starch in order to determine the location of the chemical modifications. Based on the results of specific enzymatic degradations, Kavitha and BeMiller [10] concluded that hydroxypropyl modification was preferentially located in amorphous regions as opposed to crystalline regions. Steeneken and Woortman [11] reported that the polymers obtained by methylation of granular starches were substituted in a more block-wise manner, while starch polymers methylated in solution were substituted in a more random fashion.

We used a similar selective degradation procedure to investigate structural differences between cationic starches

* Corresponding author. Tel.: +33-3-2691-3495; fax: +33-3-2691-3596.
*E-mail address:* christophe.bliard@univ-reims.fr (C. Bliard).

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obtained by different processes. This paper describes the results of enzymatic degradation α,β-amylase, amyloglucosidase and isoamylase on cationic starches with various degrees of substitution, obtained from both dry process and molten medium.

β-Amylase is an exoenzyme, which degrades α-(1-4)-D-glucopyranoside molecules by cleaving maltose units from the non-reducing ends. The action of the enzyme is stopped by the presence of α-(1-6) branch or substituting groups on α-(1-4) glucose residues. The resulting products of β-amylase actions on polysaccharides macromolecules are maltose and β-limit dextrins. Amyloglucosidase is also an exoenzyme cleaving α-(1-4) bounds from the non-reducing end of the polysaccharides chain with liberation of glucose This enzyme hydrolyses α-(1-6) as well, but at a much lower rate. Isoamylase specifically cleaves branching α-(1-6) bonds (5% in wheat amylopectin and less than 1% in amylose) releasing linear poly-α-(1-4) chains from starch branched polysaccharides. α-Amylase is an endoenzyme cleaving α-(1-4) bonds of starches with liberation of linear oligosaccharides and α-(1-6) branched dextrins. The hydrolysis products of starch are characterised by their reducing power, measured as dextrose equivalent.

A comparative degradation kinetics study between modified and non-modified starches obtained in molten medium was undertaken in order to determine the influence of the chemical modification on the enzyme affinity by determination of the constants $K_M$ and $V_{max}$.

## 2. Materials and methods

### 2.1. Polysaccharides samples

Native wheat starch (12.5% humidity) was provided by Chamtor, Bazancourt, France. Commercial cationic wheat starches synthesised by dry process with various degrees of substitution [(DS) 0.015, 0.05, 0.08 and 0.18] were provided by Crespel and Dieters, Ibbenbüren, Germany. The fused, non-modified and hydroxypropyltrimethylammonium modified starches, obtained in glycerol–water plasticised molten medium as previously described [4], are listed in Table 1. The plasticiser ratio was 30% in all formulations. They are divided into three categories as follows: group A (15% w/w glycerol and 15% w/w water), group B (7% w/w glycerol and 23% w/w water) and group C (30% water).

The dialysis tubes used to purify the β-limit dextrins after the enzymatic degradation with β-amylase were purchased from Membranes Filtration Products Inc., Seguin, TX, USA.

### 2.2. Viscosity measurement

Viscosity measurement was carried out at 25 (+/−0.1) °C with a 100 mm length and 1 mm diameter capillary using an AVS 400 semi-automatic viscosimeter from Shott, Mainz, Germany. The products were suspended in a 1 M KOH solution for 24 h followed by magnetic stirrings for another 24 h. KOH was used to limit the electrostatic interactions between the polyelectrolyte chains. The solutions were then filtered at 0.2 μm. The concentrations of the solutions were in the 1–5 g/l range. Five consecutive measurements were carried out on each sample. The measurements were performed at five different concentrations starting with the lower. The results were within 3% relative accuracy.

### 2.3. Substitution degree

Modified cationic starches and β-limit dextrins are characterised by their DS corresponding to the average number of hydroxypropyltrimethylammonium cationic groups, branched through ether bonds per glucose monomer. DS were determined by proton NMR or elementary analysis as previously described [4].

### 2.4. Measurement of reducing sugar

The enzymatic hydrolysis rates were determined by the colorimetric method developed by Park–Johnson and modified by Porro et al. [12]; maltose, β-limit dextrins and linear oligosaccharides were analysed by measuring the reducing power obtained after sample degradation.

A 10 μl aliquot of the solution containing the reducing sugar (2 μl in case of maltose) was added to 10 μl of 1 N NaOH and 380 μl of distilled water. 200 μl of a 0.1% w/v potassium ferricyanide solution and 200 μl of a 0.065% w/v potassium cyanide, 0.92% w/v sodium bicarbonate and 0.48% w/v sodium carbonate solution were added to this mixture. The resulting mixtures were incubated at 100 °C for 15 min, then left for 10 min at room temperature before adding 1000 μl of 0.3% w/v ammonium ferric sulphate in a 50 mM sulphuric acid solution. 15 min after the addition of this last solution, the absorption was recorded at 715 nm

Table 1
Plasticiser content of groups A, B and C cationic starches obtained by molten medium

| | Sample | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A-1 | A-2 | A-3 | A-4 | A-5 | A-6 | B-1 | B-2 | C-1 | C-2 |
| % Glycerol (G) | 15 | 15 | 15 | 15 | 15 | 15 | 7 | 7 | 0 | 0 |
| % Water (W) | 15 | 15 | 15 | 15 | 15 | 15 | 23 | 23 | 30 | 30 |
| DS | 0.020 | 0.028 | 0.034 | 0.045 | 0.130 | 0.160 | 0.02 | 0.045 | 0.02 | 0.045 |

with a spectrophotometer UVIKON, Milan, Italy. The results were obtained with 5% accuracy.

The glucose was measured using the Trinder enzymatic method by adding 1 ml of the Trinder reagent (Sigma Diagnostics, St. Louis, MO, USA) to a 150 μl aliquot of the mixture to analyse. The absorption was recorded at 505 nm with 5% accuracy.

### 2.5. β-Amylase degradation

Twenty-five milligrams of samples were dispersed in 10 ml of pH 4.8, 50 mM sodium acetate buffer stabilised with 0.01% w/v $NaN_3$. Ten units of powdered β-amylase (Sigma, A 7130) were added and the mixture was incubated at 20 °C for 24 h. The enzyme was then inactivated by heating the samples at 100 °C for 15 min. The carbohydrate content of each fraction was determined by the Park–Johnson method as described above.

### 2.6. Glucose liberation

Twenty-five milligrams of samples were dispersed in 25 ml of pH 4.5, 50 mM sodium acetate buffer stabilized with 0.01% w/v $NaN_3$. Ten units of amyloglucosidase (Sigma, A 7420) were then added, and the mixture was incubated at 55 °C for 3 h. The enzyme was then inactivated at 100 °C for 15 min. Analysis of the liberated glucose was determined by the Trinder method as described above but using normalized glucose solutions.

### 2.7. Isoamylase degradation

Cationic wheat starches from both dry process and molten medium were debranched using isoamylase (Sigma, I 2758) in a buffered solution, stabilised with 0.01% w/v $NaN_3$. 25 mg samples were dispersed in 10 ml of pH 3.5, 0.2 M sodium acetate buffer. Ten units of isoamylase units were added, and the mixture was incubated for 24 h at 45 °C. The enzyme was then inactivated by heating the reaction mixture at 100 °C for 15 min. The reducing power was determined for each sample using the Park–Johnson method as described above.

### 2.8. Enzyme kinetics and estimation of the $K_M$ and $V_{max}$

Kinetics were analysed on native, non-modified melted starch and two samples from group A A-1 (DS 0.02) and A-5 (DS 0.13) for β-, α-, and isoamylase. The incubation time for each sample was determined using 0.1 and 0.25 enzyme units with 0.6% substrate. The affinity constant and the catalysis rate of the enzymes was then determined for each substrate by measuring the reaction evolution after enzyme pre-incubation, using 0.1 enzyme unit at different initial concentrations (0.2, 0.6, 1 and 2% w/v).

The reducing power was determined by the Park–Johnson method as described above. The kinetic parameters ($V_{max}$: *maximal rate and* $K_M$*: Michaelis constant*) were obtained using the *Lineweaver and Burk* mode of representation [13]. The values of $-1/K_M$, representing the enzyme affinity for the substrate, were obtained from the graphs where the curves intercept the 1/[S] axis. The values of $-1/V_{max}$ were obtained from the graphs where the curves intercept the $1/U$ axis. For each series of amylolytic degradation, measurements were repeated at low, high and mean DS with a relative difference of less than 10%.

## 3. Results and discussion

### 3.1. β-Amylolytic degradation

The results of the β-amylase degradation on cationic starches from dry process and molten medium showed that the two starch types followed two different degradation modes.

The enzymatic degradation ratio obtained with the dry process products displayed a linear decrease as DSs increased, suggesting that the increase in the number of cationic groups decreases the enzymatic degradability as expected from a regular modification pattern. The results of group A starches enzymatic degradation (Figs. 1 and 2) show that the levels of degradability were lower than the ones obtained with the dry process samples at the lowest DSs (33% less degradation than with non-modified starches at DS 0.02). This result suggests that some of the chemical grafting happens on external α-(1-4) chains. The enzymatic action was not affected when the number of cationic groups were increased up to 0.13. The degradation level remained close to 30% on a wide DS range (0.02–0.13), decreasing down to 10% only at DS greater than 0.13. This suggests that the subsequent modifications occur almost exclusively on the internal chains beyond the initial modification. These results indicate that the chemical modification in molten medium exhibits a more specific pattern than in dry process.

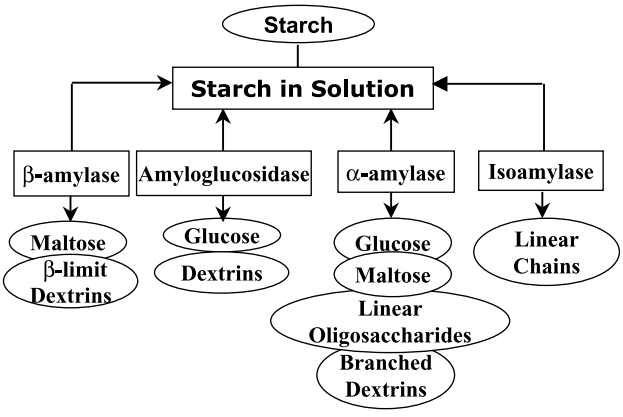


Fig. 1. Starch enzymatic hydrolysis scheme.

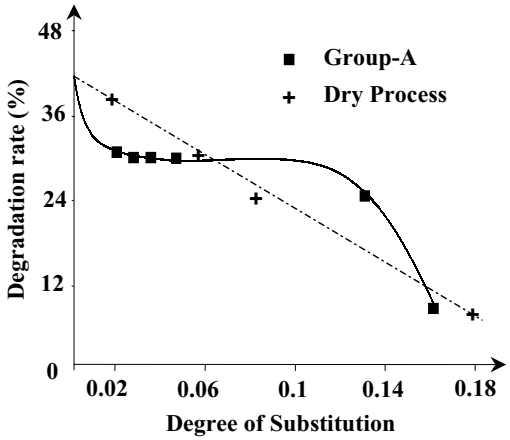


Fig. 2. β-Amylolytic degradation of group A and dry process samples.

### 3.1.1. NMR spectra

The NMR spectra of sample A-5 (DS 0.13) and the corresponding β-limit dextrin are shown in Fig. 3. The spectrum of the A-5 limit dextrin (Fig. 3B), shows an increase of the integration values for both α-(1-6) branched glucose H-1 and HPTMA protons as compared to the values from A-5 sample spectrum (Fig. 3A), this increase being proportional to the degradation ratio.

The DS value is obtained by comparing either the relative integrations of the quaternary trimethylammonium protons (nine protons at 3.35 ppm) or from the integral values of the C**H**(OH) proton from the HPTMA group at 4.6 ppm, with those of the anydroglucose anomeric protons (one proton, from 4.8 to 5.9 ppm). The ratio of 2-O- to (3-O + 6-O)-substitution (Tables 2 and 3) was calculated by comparing the integral value of the 2-O-substituted anhydroglucose residue H1 signal shifted downfield to 5.77 ppm with the one of HPTMA protons signals, confirming both the degradation level and the retention of all cationic groups on the β-limit dextrin.

Table 2
DS and substitution percentage in position 2 of group Astarch samples and of the corresponding β-limit dextrins as measured by $^1$H NMR

| Sample | Before degradation | | After degradation | |
|---|---|---|---|---|
| | DS | Substitution percentage in position 2 | DS | Substitution percentage in position 2 |
| A-1 | 0.02 | 29.38 | 0.027 | 30.46 |
| A-2 | 0.028 | 27.62 | 0.035 | 30.00 |
| A-3 | 0.034 | 26.66 | 0.046 | 27.43 |
| A-4 | 0.045 | 25.00 | 0.059 | 25.22 |
| A-5 | 0.13 | 20.00 | 0.176 | 20.00 |

Table 3
Maximum rate ($V_{max}$) and Michaelis constant ($K_M$) obtained after β-amylase pre-incubation on native, melted, A-1 and A-5 starch samples

| Sample | $V_{max}$ (mg/l min) | $K_M$ (g/100 ml) |
|---|---|---|
| Native starch | 1.42 | 0.110 |
| Melted starch | 2.04 | 0.095 |
| A-1 | 1.92 | 0.155 |
| A-5 | 2.08 | 0.178 |

### 3.1.2. Viscosity measurement

The viscosity measurements were performed on the β-limit dextrins in order to observe the influence of the β-amylase degradation on the molecular size. Fig. 4 displays the intrinsic viscosity values of various starch samples: native (N), fused under standard conditions (M) and two cationic starch samples with low and high DS (A-1 and A-5) with the corresponding β dextrins. The results indicate that the melting process causes a 63% drop in viscosity whereas β amylolytic degradation results in only a 23% drop in viscosity for both non-modified starch samples. Chemically modified β dextrins display slightly higher values depending on the DS (18% for A-1 and 14% for A-5).

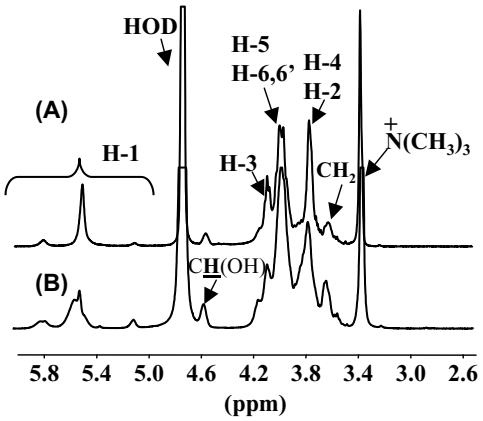


Fig. 3. $^1$H NMR spectra of sample A-5 (A) and its corresponding β-limit dextrin (B). 15 mg samples were dissolved in 100 μl NaOD and 500 μl $D_2O$. The spectra were recorded at 313 K with 124 scans.

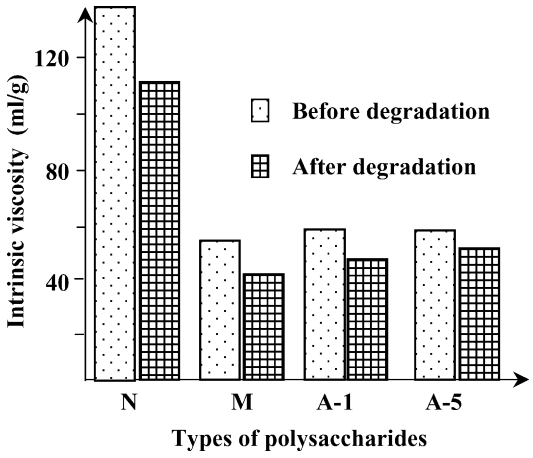


Fig. 4. Intrinsic viscosity measurements of native (N), non-modified melted starches (M) and two modified samples A-1 and A-5 before and after β-amylolytic degradation.

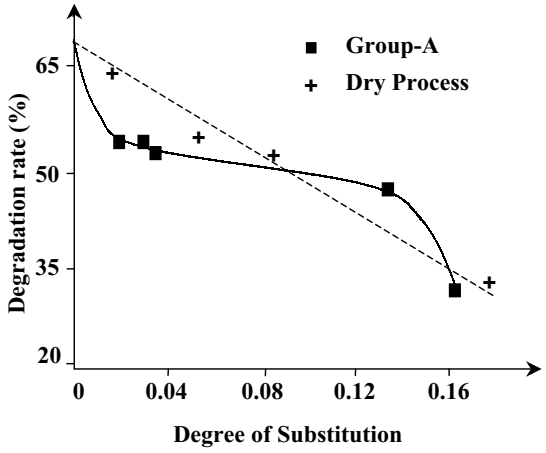


Fig. 5. Amyloglucosidase degradation of group A and dry process starches.

### 3.2. Amyloglucosidase degradation

A 67% degradation value was obtained by action of amyloglucosidase on both unmodified native and fused starch (Figs. 5 and 6).

As for the β-amylolysis, the amyloglucosidase degradation of cationic starches obtained by dry process display a gradual decrease with increasing modification, whereas the group A starch degradation is lower (48% versus 65%) at low DSs and is constant up to DS 0.13. Since amyloglucosidase is also an exoenzyme, these values tend to confirm the results obtained with β-amylolysis, that the distribution of substituting cationic groups grafted on wheat starch in molten medium are located close or beyond α-(1-6) branched glucose of the external chains.

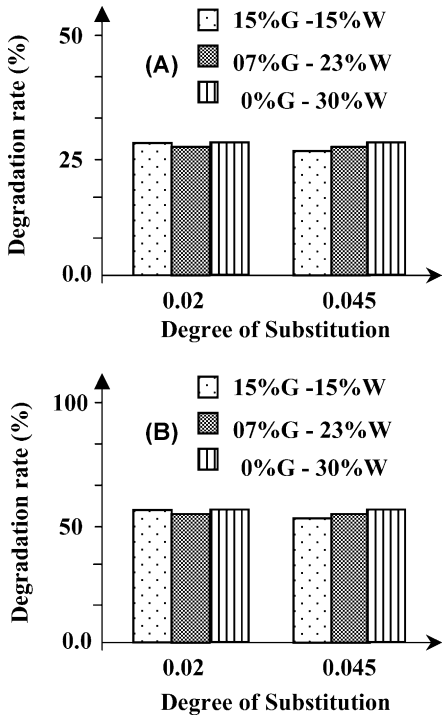


Fig. 6. Influence of various plasticising conditions (Table 1) on β-amylase (A) and amyloglucosidase degradation (B).

### 3.3. Influence of plasticising conditions

The influence of three different plasticiser amounts and types in the molten-medium chemical modification on the enzymatic degradation was studied on both exoenzymes (β-amylase and amyloglucosidase). Both glycerol and water plasticise the formulation. Three formulations were used (Table 1) A-1, A-4, B-1, B-2, C-1 and C-2. The two DS values (0.02 and 0.045) were chosen because they correspond to the extreme DS values showing a similar degradation ratio in both Figs. 2 and 5. The degradation ratios obtained in all formulations are at the same level, showing that the cationic group distribution within the macromolecules is very similar in all molten medium products.

### 3.4. α-Amylase degradation

The enzymatic degradation of native, melted, group A and dry process starches using α-amylase was undertaken. The results are displayed in Fig. 7. The degradation profiles for both dry process and melted cationic starches were strikingly similar to the ones obtained with both exoenzymes (β-amylase and amyloglucosidase). An increase of DS, from 0.02 to 0.13, did not seem to influence the enzymatic degradability level of molten medium cationic starches. But increasing the DS from 0.13 to 0.16 led to a 50% drop in the degradation level. Since α-amylase is an endoenzyme degrading regular α-(1-4) chains, this result tends to indicate that in molten medium, the linear parts of internal chains are less susceptible to chemical modification and that these modifications are concentrated near the branching zones.

### 3.5. Isoamylase degradation

The influence of the cationisation on the degradation rate with isoamylase was studied with both commercial product

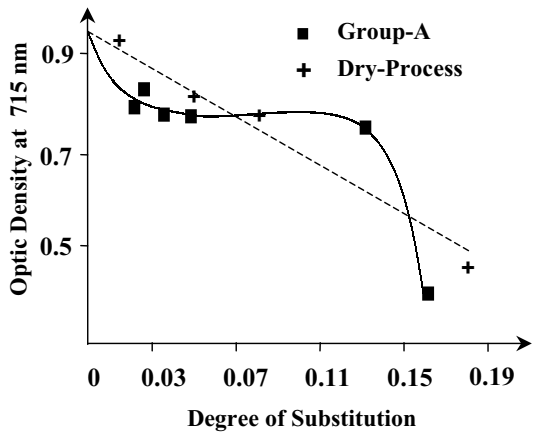


Fig. 7. α-Amylase degradation of group A and dry process starches.

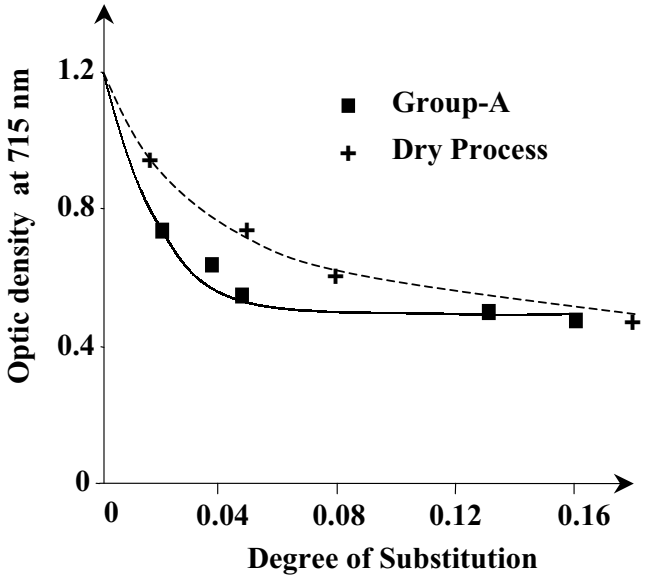


Fig. 8. Isoamylase degradation of group A and dry process starches.

and group A starches. The comparative results (Fig. 8), indicate that dry process samples follow a smooth dumping of the enzymatic activity with increasing DS whereas group A starches show a more brutal drop of the degradation level up to DS 0.05 (50%). But for DSs higher than 0.05, there appears to be no further influence on the enzymatic activity in the studied DS range. This binary degradation mode for less than and greater than DS 0.05 using isoamylase seems to show that at low DSs (<0.05), the chemical modification happens selectively in the vicinity of the branching area, hindering the action of isoamylase. Even when increasing the number of cationic groups from DS 0.05 to 0.16, the enzyme continue to debranch the same amount of linear chains. The isoamylase degraded A-5 proton NMR spectrum confirms the presence of reducing end H-1 protons and 2.5% remaining α-(1-6) glucose H-1 proton.

### *3.6. Estimation of kinetic parameters of enzymatic degradation with α-, β-amylase and isoamylase enzyme*

Figs. 9–11 display the comparative results of kinetics obtained with native, non-modified melted starch and two cationic starch products at low and high DS A-1 and A-5.

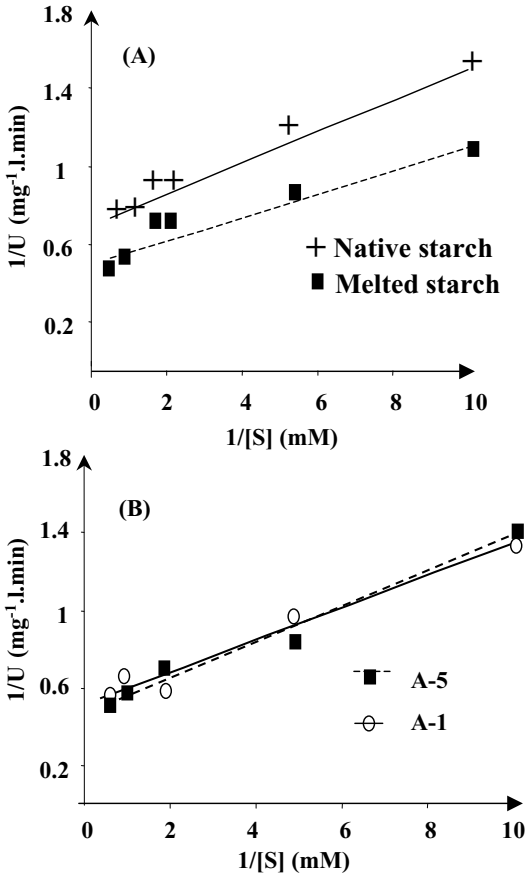


Fig. 9. Determination of β-amylase kinetic parameters $K_M$ and $V_{max}$ for native and non-modified melted starches (A); samples A-1 and A-5 (B). *U* corresponds to the quantity of liberated maltose (mg/l min). [S]: substrate concentration in the buffer medium.

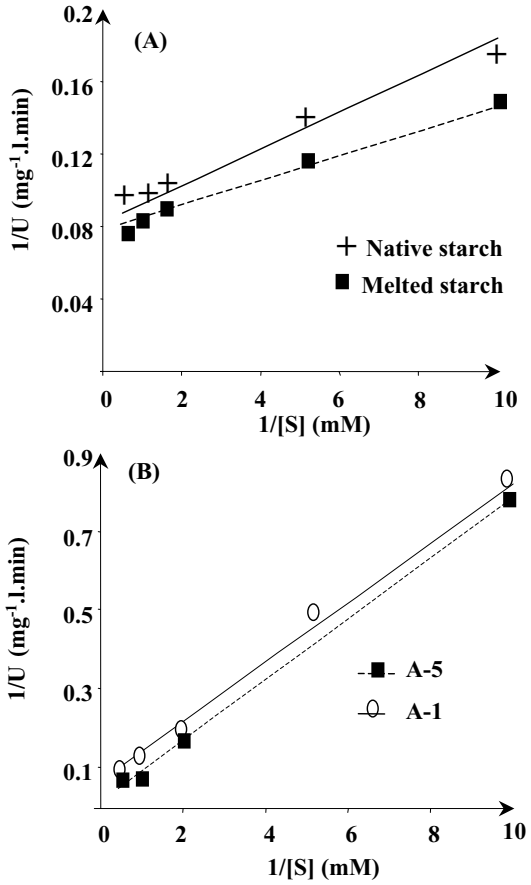


Fig. 10. Determination of α-amylase kinetic parameters $K_M$ and $V_{max}$ for native and non-modified melted starches (A); samples A-1 and A-5 (B). *U* corresponds to the quantity of liberated reducing end (normalised as maltose) in mg/l min. [S]: substrate concentration in the buffer medium.

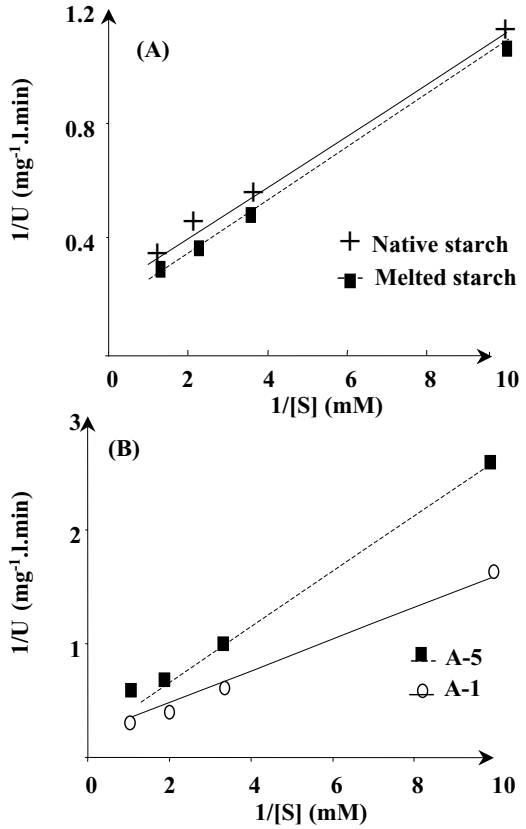


Fig. 11. Determination of isoamylase kinetic parameters $K_M$ and $V_{max}$ for native and non-modified melted starches (A); samples A-1 and A-5 (B). *U* corresponds to the quantity of liberated reducing end (normalised as maltose) in mg/l min. [S]: substrate concentration in the buffer medium.

### 3.6.1. β-Amylase degradation

The $K_M$ for both non-modified native and melted starches were similar (0.11 and 0.095%). $K_M$ values of group A starches are slightly higher, suggesting a slight decrease of the enzyme affinity for the modified substrate. Furthermore, when the results obtained for starch modified in molten medium are compared with those of the reference (melted starch), it can be noted that the cationisation, though weak, seems to affect the affinity of the enzyme for its substrate. There was a 40% increases of $V_{max}$ with melted starch but no further increase was noticed with chemical modifications.

### 3.6.2. α-Amylase degradation

The results obtained with α-amylase (Table 4) are similar to those of β-amylase, the melting process having no influence on the enzyme affinity for non-modified starches. However, with increasing chemical modification, the α-amylase affinity for the substrate decreases greatly, much more so than that observed with β-amylase. These results confirm that the chemical modification takes place preferentially within the macromolecule and not at the non-reducing ends. The melting process does not appear to have a noticeable influence on the $V_{max}$. In contrast, the $V_{max}$ doubles with the chemical modification (+103% from melted starch to A-5).

Table 4
Maximum rate ($V_{max}$) and Michaelis constant ($K_M$) obtained after α-amylase pre-incubation on native, melted, A-1 and A-5 starch samples

| Sample | $V_{max}$ (mg/l min) | $K_M$ (g/100 ml) |
|---|---|---|
| Native starch | 12 | 0.123 |
| Melted starch | 12.82 | 0.090 |
| A-1 | 22.40 | 1.724 |
| A-5 | 26 | 5 |

Table 5
Maximum rate ($V_{max}$) and Michaelis constant ($K_M$) obtained after isoamylase pre-incubation on native, melted, A-1 and A-5 starch samples

| Sample | $V_{max}$ (mg/l min) | $K_M$ (g/100 ml) |
|---|---|---|
| Native starch | 4.62 | 0.418 |
| Melted starch | 5.62 | 0.491 |
| A-1 | 5.64 | 0.781 |
| A-5 | 7.32 | 1.785 |

### 3.6.3. Isoamylase degradation

In the case of the isoamylase (Table 5), the melting process seems to causes a slight decrease in the debranching enzyme affinity for the substrate, the decrease becoming more pronounced when the chemical modification increases. The melting process causes a 21% increase in the $V_{max}$ and a further increase of 30% is observed with the chemical modification from melted starch to A-5.

## 4. Conclusions

Enzymatic degradation of cationic starches, synthesised by dry process and under molten conditions displayed distinct degradation profiles. The results obtained with exoenzymes show that the chemical modification first happens on some external α-(1-4) chains with a more specific pattern in molten medium products. With the former, much lower degradability levels were obtained at the lowest DSs. Nevertheless, these degradability levels remain constant until DS 0.13, indicating that further chemical modifications occur on internal chains towards the reducing end, exclusively beyond the initial modifications. α-Amylase degradation displayed identical profiles, suggesting that regular α-(1-4) internal as well as external chains, are less susceptible to chemical modification. Chemical modification had a very strong influence on isoamylase activity, particularly in molten medium products, showing that the grafted groups are located in the close vicinity of the α-(1-6) branched glucose residues. Chemical modification had an impact on the kinetic parameters of each enzyme ($K_M$ and $V_{max}$), particularly on α-amylase. Further investigation into the structural characterisation of cationic starches, using NMR spectroscopy, are in process to determine the exact location of these grafted groups.

## Acknowledgements

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