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Original article

Characterization of caseinomacropeptides released from renneted raw and UHT treated milks

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Summary — The kinetics of release of the glycosylated and carbohydrate-free forms of caseinomacropeptide (CMP) was studied in renneted raw and UHT (140 °C for 10 s) milks. The new chromatographic method utilized allowed quantitative determinations of both molecular forms. UHT treatment leads to a 40% decrease of the final content in glycosylated forms compared to the value determined in raw milk. The results obtained show that carbohydrate-free κ-casein constitutes 52% of whole κ-casein. When milk is UHT treated, β-lactoglobulin only seems to influence the release of the glycosylated form. Nevertheless, no conclusion can be drawn concerning the localization in the micellar structure of both molecular forms of κ-casein.

milk / coagulation / rennet / glycosylated CMP / carbohydrate-free CMP / thermal treatment / UHT treatment

Résumé — Caractérisation des caséinomacropeptides libérés lors de la coagulation par la présure de laits cru et UHT. *Grâce à une nouvelle méthode chromatographique permettant la séparation du caséino-macropeptide (CMP_t) résultant de l'action de la présure sur la caséine κ, les cinétiques de libération des formes glycosylées et non glycosylées du CMP_t à partir du lait cru et du même lait traité UHT, chacun soumis à l'action de la présure, ont pu être quantifiées. Le traitement UHT conduit à une diminution d'environ 40% de la teneur finale en CMP glycosylé par rapport à la valeur déterminée sur le lait cru. Les résultats obtenus précisent la répartition entre formes glycosylées (42%) et non glycosylées (58%) de la caséine κ. Ils démontrent également une influence très préférentielle de la β-lactoglobuline sur la libération des seules formes glycosylées de cette caséine lors du chauffage du lait. Mais ils ne permettent pas de conclure quant à la localisation de ces formes glycosylées au sein de la structure micellaire.*

lait / coagulation / présure / CMP glycosylé / CMP non-glycosylé / traitement thermique / UHT

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INTRODUCTION

Rennet coagulation is a result of a 2-phase mechanism: enzymatic splitting of one of the milk caseins, κ-casein, followed by an aggregation of the resulting para-casein micelles into a network in which calcium salts are involved. κ-casein is a heterogeneous protein with 2 main genetic variants, A and B, each having at least 7 other molecular forms differing in their glycosylation level (Walstra and Jenness, 1984). The proportion of carbohydrate-free molecules of κ-casein is estimated at between 33% (Walstra and Jenness, 1984) to 40% (Vreeman *et al,* 1986). The more recent model proposed for the structure of casein micelles (Schmidt, 1982) shows most of the κ-casein molecules on the outside; the micelles have a hairy surface due to their protruding C-terminal ends (Walstra and Jenness, 1984). Numerous recent studies (Yoshikawa *et al,* 1978; Kudo *et al,* 1979; Walstra, 1979; McGann *et al,* 1980; Mehaia and Cheryan, 1983) favour an external localization of glycosylated κ-casein, while Wheelock and Knight (1969) and Sinkinson and Wheelock (1970) conclude that the localization is internal.

Heating of milk induces formation of a β-lactoglobulin – κ-casein complex which reduces the accessibility of the Phe_{105}–Met_{106} bond to chymosin (Dalgleish, 1990; Reddy and Kinsella, 1990). Consequently, the final amount of the released C-terminal part of κ-casein (caseinomacropeptide, CMP_t) is reduced proportionally to the intensity of the heat treatment previously applied to milk.

Using a new chromatographic method recently proposed by Léonil and Mollé (1991), we have shown that heating milk to 140 °C for 10 s led to an 18% decrease in CMP_t release by chymosin (Ferron-Baumy *et al,* 1991).

As the method of Léonil and Mollé (1991) allowed separation of the carbohydrate-free form of CMP_t from the glycosylated forms, the purpose of the present paper was to quantify the release of these 2 groups of κ-casein fragments in renneted raw and UHT treated milks.

MATERIALS AND METHODS

Milk preparation

About 40 l of bulk whole raw milk, obtained from an industrial dairy plant (Bridel, L'Hermitage, France) were skimmed at 50 °C with a Westfalia DD 100 Z separator (Chateau-Thierry, France). Direct UHT treatment (140 °C for 10 s) was performed in a steam sterilizer (Laguilharre, Rueil-Malmaison, France).

Determination of caseinoglycomacropeptide (CMP)

CMP contents of milks to which were added 50 μl of rennet extract containing 520 mg of chymosin per l for 100 ml of sample were determined at intervals after renneting at the initial milk pH, as indicated by Léonil and Mollé (1991) by FPLC (Mono S column) of its pH 4.6 filtrate. For each point, the coefficient of variation was estimated to be 4%.

Characterization of the 2 fractions of CMP_t

Ten samplings of the fractions corresponding to each peak of CMP_t obtained as described by the method of Léonil and Mollé (1991) were pooled and dried in a "Speed-Vac" concentrator (Savant Inst, USA). Both dried fractions were then dissolved in 500 μl of bidistilled water. On each fraction, 3 determinations were then made: firstly, a RP–HPLC analysis on a Vydac column 218 TP 54 as described by Léonil and Mollé (1991);

and secondly, determination of the sialic acid content according to Warren (1959); thirdly, a treatment with 0.5 N NaOH at room temperature for 10 h was applied to each fraction. The release of α-amino crotonyl residue was measured by following the increase in absorbance at 241 nm using a UVIKON 810 (Kontron Instruments) spectrophotometer according to the procedure of Carubelli *et al* (1965).

Preparation of CMP_t from raw and UHT milks by preparative chromatography

To 200 ml of each milk were added 4 ml of a rennet solution (5 ml of rennet extract diluted in 200 ml of 25 mmol.l^{-1}, pH 5.2 piperazine buffer). After enzyme inactivation by 15 ml of 0.2 N NaOH, both samples were treated at 60 °C during 15 min, then acidified to pH 4.6 and filtered on Whatman 42. Filtrates obtained from raw or UHT milks were analyzed on a S-Sepharose Fast Flow column containing 300 ml of exchange resin. The chromatographic analysis was performed with a Biopilot apparatus (Pharmacia, St-Quentin-en-Yvelines, France). Operating conditions were: flow rate: 1.2 l.h^{-1}, eluant: KCl 20 mmol.l^{-1}, pH 2. Elution was obtained with a step-by-step NaCl gradient. Absorbance was recorded at 214 nm. Three fractions were obtained from each filtrate, collected, dialysed on a Spectra/por PM 1 000 membrane (Poly Labo, Colmar, France) and then freeze-dried.

RESULTS

Characterization of both CMP_t fractions obtained by chromatography

The elution profile observed on Mono S column with a pH 4.6 filtrate obtained from renneted raw skim-milk is shown in figure 1. CMP_t is eluted in 2 peaks, noted 1 and 2. The Mono S column utilized in this study allowed a better separation of these 2 peaks than that used by Léonil and Mollé (1991). Identity of amino acid composition between these 2 fractions and reference CMP_t was checked.

Figure 2 compares the RP–HPLC profiles obtained from whole CMP_t and from isolated fractions 1 and 2. As already observed by Léonil and Mollé (1991), the A and B genetic variants of the non-glycosylated forms of CMP_t clearly appear in the elution profiles of whole CMP_t (fig 2c) and fraction 2 (fig 2b). On the other hand, the elution profile of peak 1 (fig 2a) shows numerous peaks which are not well separated and with retention times that are somewhat shorter than those of the non-glycosylated molecular forms. Comparative study of figure 2 chromatograms leads to the hypothesis that peak 1 may gather glycosylated forms of κ-casein macropeptide. This hypothesis was confirmed by sialic acid determination in fractions 1 and 2. They contained 0.13 and 0.006 μmol.mg^{-1} of freeze-dried powder, respectively. Moreover, the alkaline treatment of each fraction led to a progressive increase of 241 nm absorbance in 6 h only for peak 1. Such a result confirms the presence of sugar derivatives bound to threonine residues through *O*-glycosidic linkages in the peptide chain (Carubelli *et al*, 1965).

Influence of heat treatment of milk on the kinetics of κ-casein hydrolysis

Figure 3 shows kinetics of release observed for both fractions of CMP_t from renneted raw and UHT milks. No significant difference between the 2 milks appears regarding to the final contents of released carbohydrate-free macropeptides (fractions 2). The found values are respectively 0.64 g.l^{-1} for raw milk and 0.65 g.l^{-1} for UHT milk. On the contrary, initial hydrolysis rates expressed in μmol.l^{-1}.min^{-1} are significantly different and equal to 6.8 for raw milk and 5.6 for UHT milk respectively. Re-

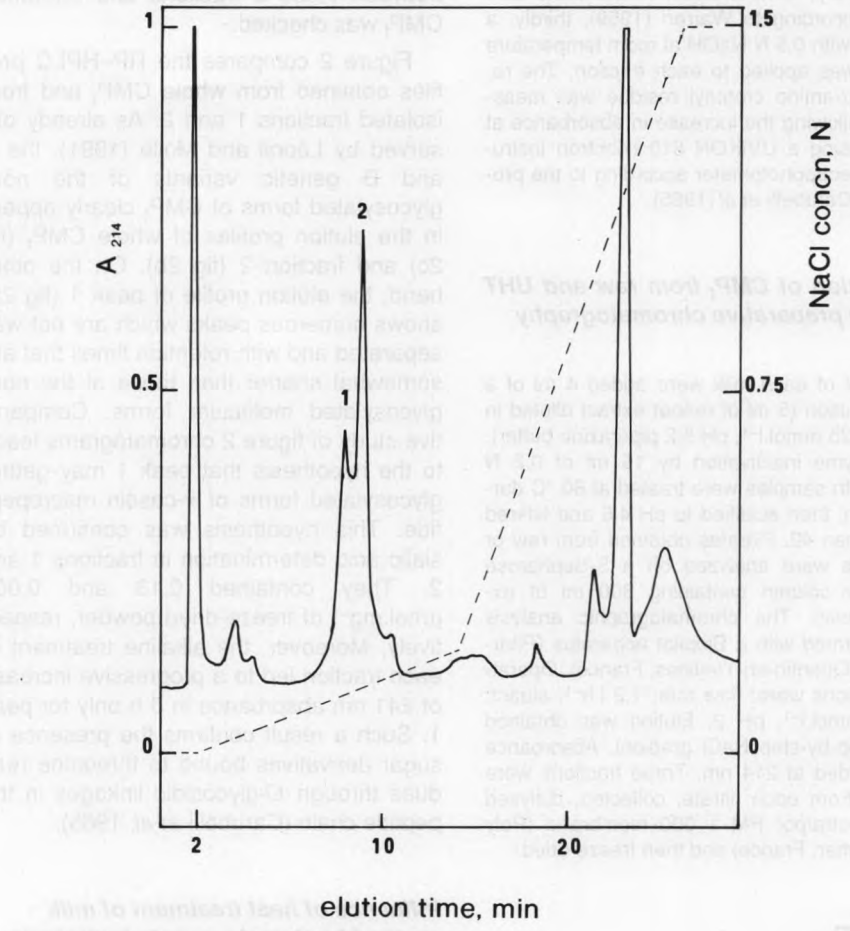

Fig 1. FPLC separation of macropeptide, by cation-exchange chromatography, obtained from pH 4.6 filtrate, using pH 2, 20 mmol.l^{-1} KCl.HCl buffer and a NaCl gradient on a Mono S column.

Séparation par FPLC, en chromatographie d'échange de cations, du caséinomacropeptide obtenu à partir d'un filtrat pH 4,6, en utilisant un tampon KCl.HCl 20 mmol.l^{-1}, pH 2, sur une colonne Mono S.

leases of glycosylated macropeptides (fractions 1) are very different. Final contents are 0.51 g.l^{-1} for raw milk and 0.31 g.l^{-1} for UHT milk. This represents a 40% decrease resulting from heat treatment.

Sialic acid determination in F_2 fractions isolated by preparative chromatography from pH 4.6 filtrates obtained from renneted raw and UHT milk (fig 4) has confirmed this value. Indeed, these F_2 fractions con-

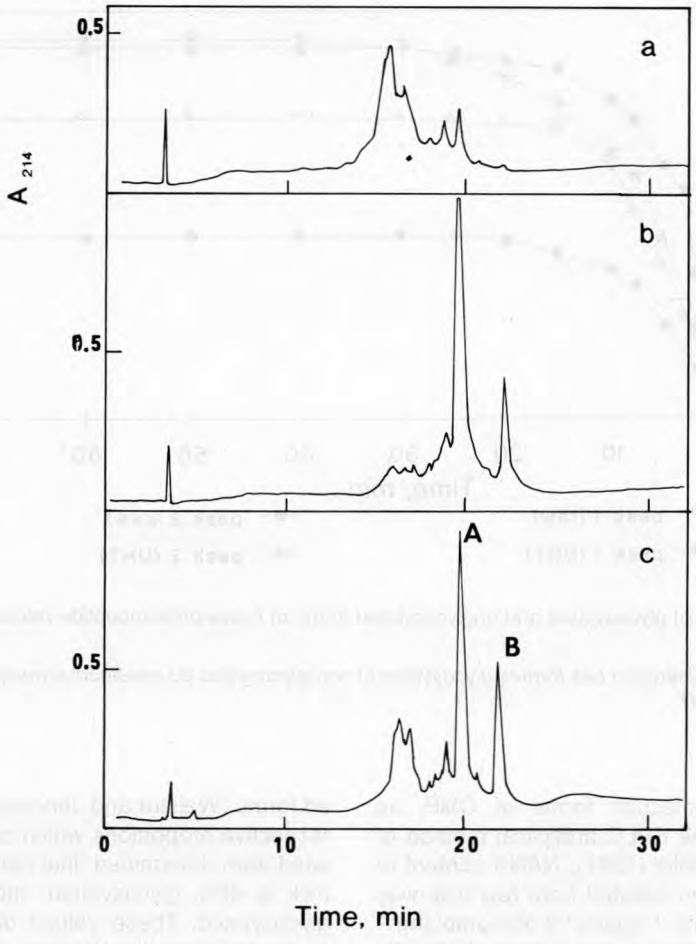

Fig 2. Elution profiles by reversed-phase HPLC on a Vydac C_{18} column (218TP54) of different fractions collected after cation-exchange chromatography. Elution was performed using a linear gradient from 8 to 80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 $ml.min^{-1}$. **a.** Elution profile of peak 1 collected after cation-exchange chromatography. **b.** Elution profile of peak 2 collected after cation-exchange chromatography. **c.** Elution profile of both the peaks collected after cation-exchange chromatography. Peaks A and B represent carbohydrate-free macropeptide A and B variants respectively.

*Profils d'élution en CLHP phase inverse sur une colonne Vydac C_{18} (218TP54) des différentes fractions collectées après chromatographie d'échange de cations. L'élution était réalisée en utilisant un gradient linéaire de 8 à 80% d'acétonitrile dans un tampon eau/acide trifluoroacétique 0,1% à un débit de 1 $ml.min^{-1}$. **a.** Profil d'élution du pic 1 collecté après chromatographie d'échange de cations. **b.** Profil d'élution du pic 2 collecté après chromatographie d'échange de cations. **c.** Profil d'élution des 2 pics collectés après chromatographie d'échange de cations. Les pics A et B représentent respectivement les variants A et B du macropeptide non glycosylé.*

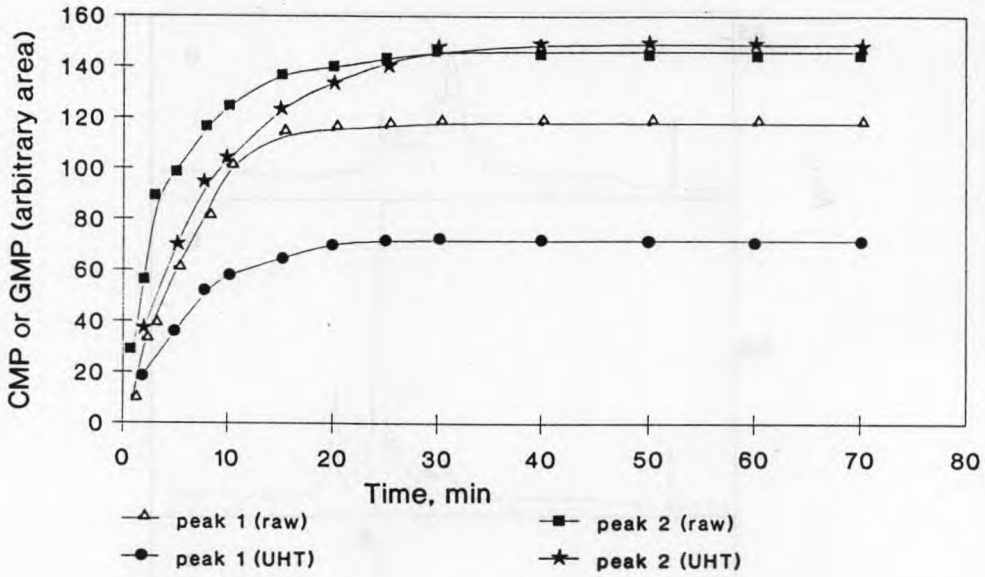

Fig 3. Kinetics of glycosylated and unglycosylated forms of caseinomacropeptide release in raw and UHT milks.

Cinétiques de libération des formes glycosylées et non glycosylées du caséinomacropeptide dans les laits crus et UHT.

tain both molecular forms of CMP_t as verified by the FPLC analytical method of Léonil and Mollé (1991). NANA content of the F_2 fraction isolated from raw milk was 0.058 $\mu mol.mg^{-1}$ against 0.032 $\mu mol.mg^{-1}$ in the F_2 fraction isolated from UHT milk. This constitutes a 45% decrease.

DISCUSSION

The new chromatographic method proposed by Léonil and Mollé (1991) allows a quantitative separation of the carbohydrate-free and glycosylated forms of CMP_t released during rennet hydrolysis of κ-casein. Taking into account a molecular weight of 6754 Da for the non-glycosylated form and an average of 7280 Da for the glycosylated forms (Walstra and Jenness, 1984), the respective proportions which can be calculated from determined final contents in raw milk is 42% glycosylated and 58% non-glycosylated. These values disagree with those indicated by Walstra and Jenness (1984): 67% and 33% respectively and by Vreeman *et al* (1986): 60% and 40% respectively. Nevertheless, careful examination of the results of Vreeman *et al* (1986) shows that NANA residues were not clearly detected in 4 κ-casein fractions constituting 17.6% by weight of that casein. Attribution of these 4 fractions to glycosylated forms by the authors can be considered as highly speculative.

Comparison of carbohydrate-free and glycosylated CMP releases in renneted raw milk shows a slower hydrolysis of the

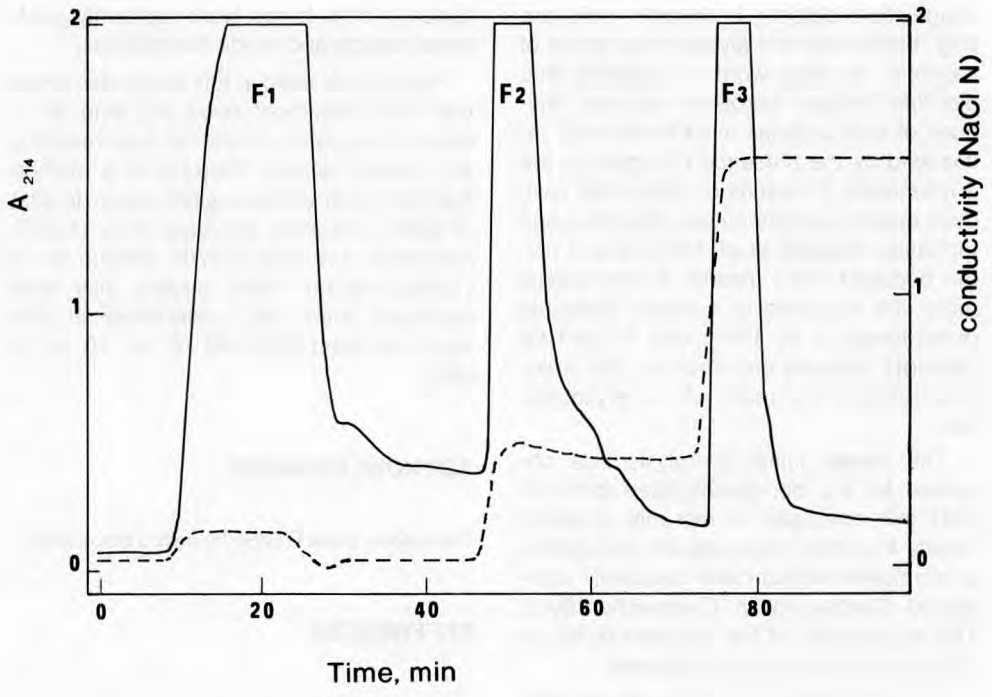

Fig 4. Elution profile of cation-exchange preparative chromatography on a S-Sepharose Fast Flow column obtained from pH 4.6 filtrate. Operating conditions were: flow rate: 1.2 $l.h^{-1}$, eluant: pH 2, 20 $mmol.l^{-1}$ KCl.HCl. Elution was obtained with a step-by-step NaCl gradient.

Profil d'élution obtenu à partir d'un filtrat pH 4,6 par chromatographie préparative d'échange de cations sur une colonne S-Sepharose Fast Flow. Les conditions opératoires étaient les suivantes : débit 1,2 $l.h^{-1}$, éluant : KCl.HCl 20 $mmol.l^{-1}$, pH 2. L'élution était obtenue à l'aide d'un gradient par paliers de NaCl.

Phe_{105}–Met_{106} linkage in glycosylated κ-casein than in its non-glycosylated forms. Such an observation has already been made by numerous authors: Sinkinson and Wheelock, 1970; Doi *et al,* 1979; Fournet *et al,* 1979; Jollès and Fiat, 1979; Addeo *et al,* 1984; Van Hooydonk *et al,* 1984. It has been attributed to a reduced accessibility to chymosin of the glycosylated sites of κ-casein which would have higher electronegativity than the carbohydrate-free forms (Van Hooydonk *et al,* 1984).

UHT treatment (140 °C for 10 s) of milk before renneting leads to a 40% decrease in glycosylated CMP release. Such a result is also in correct agreement with our former results (Ferron-Baumy *et al,* 1991) describing an 18% decrease in whole CMP release for the same heat treatment and a 42% proportion of glycosylated CMP determined in this study. On the other hand, the identical final contents in carbohydrate-free CMP of renneted raw and UHT milks lead to the conclusion that the β-lactoglobulin-κ-casein complexes which cause rennet

coagulation defects in heated milk are only formed with the glycosylated forms of κ-casein. In other words, it appears that disulfide bridges between cysteinyl residues of both proteins must be strongly influenced by the presence of sugars in the environment. Presence of hydrophilic residues around cysteine favors disulfide bond formation (Muskal *et al,* 1990) and it can be deduced from recent 3-dimensional molecular modeling of κ-casein molecule (Kumosinski *et al,* 1991) that 11 and 88 cysteinyl residues are close to 133 threonine residue, the main site of glycosylation.

The slower initial hydrolysis rate observed for the non-glycosylated forms in UHT milk compared to raw milk probably results from the increased electronegativity of micelles which have covalently complexed β-lactoglobulin (Dalgleish, 1990). The accessibility of the Phe-Met bonds to chymosin is consequently reduced.

This study does not allow the micellar localization of both molecular forms of κ-casein to be determined. β-lactoglobulin–κ-casein complex formation caused by UHT treatment with only glycosylated forms could argue in favour of an external micellar localization. Such glycosylated κ-casein should be more accessible to β-lactoglobulin than the non-glycosylated κ-casein. Such a conclusion would be in agreement with the hypotheses put forward by Yoshikawa *et al* (1978), Kudo *et al* (1979), Walstra (1979), McGann *et al* (1980), Mehaia and Cheryan (1983). However, β-lactoglobulin could also penetrate the casein micelle which has a very porous structure as shown by Ribadeau Dumas and Garnier (1970). The reduced initial rates of hydrolysis by chymosin observed wit both molecular forms of κ-casein in UHT milk could also indicate an external localization of the glycosylated forms but the same results could be obtained if both forms were uniformly scattered outside and inside the micelles.

Results obtained in this study also show that UHT treatment does not lead to κ-casein hydrolysis similar to that resulting from rennet activity. Release of a peptide fragment with a composition close to that of CMP_t observed by Alais *et al* (1967), Nakanishi and Itoh (1970), Khalifa *et al* (1985) requires more severe milk heat treatment than that undertaken in this study (at least 100–140 °C for 10 to 30 min).

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