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Carbohydrate Research 340 (2005) 1780–1788
Carbohydrate RESEARCH

Characterisation of a 1,4-β-fucoside hydrolase degrading colanic acid

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Received 19 January 2005; received in revised form 27 April 2005; accepted 7 June 2005
Available online 1 July 2005

Abstract—A novel colanic acid-degrading enzyme was isolated from a mixed culture filtrate obtained by enrichment culturing of a compost sample using colanic acid as carbon source. The enzyme was partially purified resulting in a 17-fold increase in specific activity. Further purification by Native PAGE revealed that the enzyme is part of a high-molecular weight multi protein complex of at least six individual proteins. The enzyme showed a temperature optimum at 50 °C while after 5 h at 50 °C and pH 7 still 70% of the total activity was left. The pH optimum was found to be pH 7. Analysis of the degradation products showed that the enzyme is a novel 1,4-β-fucoside hydrolase that liberates repeating units of colanic acid with varying degrees of acetylation. K_m and V_{max} of the enzyme were determined against the native substrate as well as its de-*O*-acetylated and depyruvated forms. Compared to the native substrate the affinity of the enzyme for the modified substrates was much lower. However, for the de-*O*-acetylated sample a dramatic increase in catalytic efficiency was observed. The native form of the substrate showed substrate inhibition at high concentrations, probably due to the formation of nonproductive substrate complexes. Removal of the acetyl groups probably prevents this effect resulting in a higher catalytic efficiency.

Keywords: Colanic acid; Exopolysaccharide (EPS); Enzyme; Fucoside hydrolase; Paper industry; Biofouling

1. Introduction

The formation of slime layers induced by biofilm formation on the surface of paper machines causes significant operational problems within the paper industry. These problems include defects in the final product like spots and holes and process downtime due to web breaks and clogging of the paper machine itself. Slime is defined as the accumulation of microbial cells immobilised and embedded in an organic polymer matrix of microbial origin, consisting of carbohydrates, proteins and possibly smaller amounts of nucleic acids and (phospho)lipids.[1] The major components of this matrix are usually exopolysaccharides (EPSs), mixed in different proportions with fibres, fines, fillers and other materials that are trapped in this polymer matrix.[2] Previous research by Verhoef et al.[3] showed the presence of the EPS colanic acid in several different paper mills within Spain and Finland. Colanic acid is commonly known to be produced by several members of the Enterobacteriaceae family.[4]

Colanic acid was first discovered by Goebel[5] in 1963 and ever since numerous studies have been carried out to elucidate the chemical fine structure to obtain a better understanding of the formation of this EPS and determine its role within a developing or mature biofilm architecture. Only recently it has been discovered that colanic acid is not necessarily a primary biofilm former, but plays an important role in the development of the complex three-dimensional structure of a biofilm.[6] However, apart from previous works done by Verhoef et al.[3] and van Speybroeck et al.[7] this EPS was never directly associated with slime problems within a paper mill environment.

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doi:10.1016/j.carres.2005.06.003

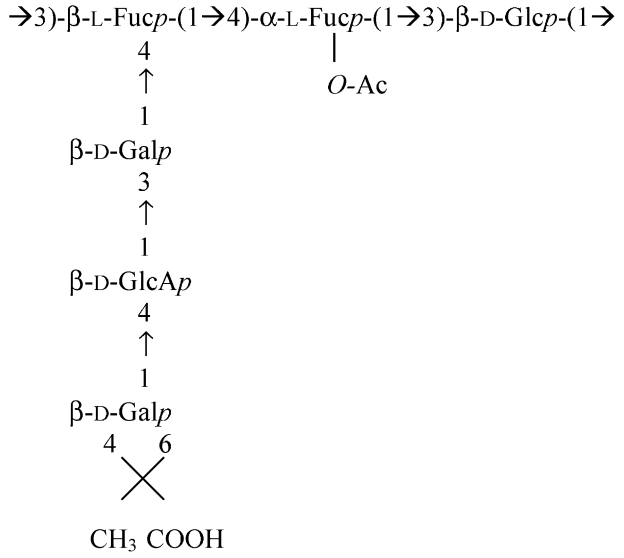

Figure 1. Structure of the repeating unit of colanic acid.[9,10]

Colanic acid is composed of fucose, galactose, glucose and glucuronic acid together with acetyl and pyruvate in molar proportions of 2:2:1:1:1:1, respectively.[8] The structure of colanic acid[9,10] is presented in Figure 1 and shows the presence of an *O*-acetyl group at position C-2 or C-3 of the nonbranched fucose and a pyruvate ketal at the terminus of the side chain of the repeating unit. It is known that colanic acid produced by different species has the same branched hexasaccharide repeating unit, but per species variation within the substituents may occur. The pyruvate moiety is either linked to position C-4 and C-6 or C-3 and C-4 of the galactose terminus of the side chain, furthermore instead of a pyruvate ketal also acetal forms of formaldehyde or acetaldehyde have been found linked to the same positions.[9]

Enzymatic degradation of colanic acid was first achieved by Sutherland[11] using a phage induced enzyme hydrolysing the →1)-α-L-Fuc*p*-(1→3)-β-D-Glc*p* linkage. Furthermore, a patent application describing the use of an enzyme, isolated from a novel *Streptomyces* strain, able to partly degrade colanic acid, has been filed by van Speybroeck et al.[7] The present paper describes the characterisation and partial purification of a novel 1,4-β-fucoside hydrolase that causes a complete conversion of colanic acid to its corresponding hexasaccharide repeating unit. Furthermore, enzymatic research revealed new insights in the *O*-acetylation pattern of colanic acid.

2. Experimental

2.1. Production of enzyme degrading colanic acid

Cultures degrading colanic acid were obtained by enrichment cultivation of a sample of compost, containing paper machine fines, chicken manure and wood bark. The enrichment medium contained 5 g/L colanic acid from a *Citrobacter* sp. (VTT E-011941, produced as described in[12]) and 6.7 g/L yeast nitrogen base (Difco) in 50 mM potassium phosphate buffer, pH 7. About 1 g of mechanically homogenised compost sample was suspended in 5 mL of physiological NaCl solution, the suspension was mixed and the solids were allowed to settle. Suspension (250 μL) was inoculated in 5 mL of enrichment medium. Sterile water was added to the reference cultures instead of compost sample. Enrichment cultures were incubated at 50 °C without mixing for 3 days. Polysaccharide-degrading activity was detected by following the decrease in the viscosity using a Brookfield DV II viscometer (+20 °C, sample size 0.5 mL). When the viscosity in the 1st enrichment culture had clearly decreased, 250 μL of culture medium was re-inoculated into 5 mL of fresh enrichment medium. After 3 day of cultivation when the viscosity of the second enrichment culture was lowered, 500 μL of the culture medium was re-inoculated into 10 mL of fresh medium for the third enrichment culture. After 1–2 days of incubation the enrichment cultures were stored in the culture medium at +4 °C for immediate use or stored in 5% glycerol at −80 °C for longer periods.

2.2. Activity assay

To follow the purification and to measure the kinetic parameters of the enzyme, the bicinchoninic acid (BCA) assay according to Meeuwsen et al.[13] was used. The substrate solution consisted of 0.5 mg/mL colanic acid in 50 mM phosphate buffer pH 7. This substrate solution was mixed with variable amounts of enzyme solution until a total volume of 100 μL and was incubated at variable temperatures. The amount of reducing sugars released upon degradation was determined using a fucose calibration curve (5–500 μM). Specific activities were expressed as U/mg protein where U stands for μmol reducing sugar released per minute. The protein content was measured according to Bradford[14] using bovine serum albumin as a standard.

2.3. Anion exchange chromatography

The crude culture filtrate was ultra-filtered (Amicon 10 kDa cut off) and washed with Millipore water (<20 μS/cm^2) followed by 25 mM Tris/HCl buffer pH = 7 and then applied on a Mono Q 5/50 GL (Amersham Biosciences) anion exchange column equilibrated with the same buffer. The protein was eluted from the column by increasing the ionic strength at a flow rate of 1 mL/min from 0 to 1 M NaCl in 20 column volumes, and fractions (0.5 mL) were collected.

2.4. Gel permeation chromatography

The pool containing enzyme activity after MonoQ separation was applied to a HiLoad 16/60 Superdex 200 Prepgrade (Amersham Biosciences) gel filtration column and eluted at 1 mL/min using 100 mM Tris/HCl buffer pH 7.0 and fractions (2 mL) were collected.

2.5. pH optimum and temperature optimum

The pH optimum was determined in McIlvaine buffers in the pH range from 3 to 8. The colanic acid solutions (1 mL; 0.5 mg/mL) were incubated with 20 μL of enzyme solution (pooled after Superdex 200 separation; 23 μg protein/mL) at 30 °C for 5 h. The temperature optimum was measured using 0.5 mg/mL colanic acid in 50 mM phosphate buffer pH 7 in the range from 20 to 80 °C. The colanic acid solutions (1 mL) were incubated with 25 μL of the same enzyme pool for 5 h. Temperature stability was measured by first heating 20 μL of the same enzyme solution at the same temperature range without substrate for 1 h, followed by addition of 1 mL 0.5 mg/mL colanic acid in 50 mM phosphate buffer pH 7 and 5 h incubation at 30 °C.

2.6. Native and SDS-PAGE

SDS-PAGE was run on a PhastGel™ Gradient 4–15% gel (Phastsystem), using low-molecular weight protein standards from Amersham Biosciences. The gel was stained using silver staining. Native PAGE was run using a BioRad MiniProtean electrophoresis unit on a 5–15% gradient PAGE gel (ready gel BioRad). Part of the gel was Coomasie stained and three unstained lanes were cut into slices of 2 mm. The sections from one lane were incubated with 1 mL 0.5 mg/mL colanic acid (30 °C, 50 mM phosphate buffer pH 7, 18 h) to determine the active protein band. The BCA reducing sugar assay as well as HPSEC were used for analysis of degradation products. The other two lanes were used for enzyme and substrate blanks. After incubation of the enzyme blank, the enzyme diffused into the buffer. This active enzyme fraction was concentrated and subjected to SDS-PAGE under denaturing conditions with and without prior reduction with β-mercaptoethanol.

2.7. Substrate modifications

Native colanic acid was obtained as described by Verhoef et al.[3] Colanic acid was de-*O*-acetylated by dissolving 60 mg of colanic acid in 25 mL 100 mM NaOH and stirring for 18 h at 4 °C. After this saponification, the de-*O*-acetylated substrate was dialysed by ultra-filtration using a 10 kDa cut off Amicon filter. The ultra filtrated solution was freeze dried to obtain the de-*O*-acetylated substrate. Colanic acid (60 mg) was depyruvated as described by Verhoef et al.[15] using autohydrolysis.

2.8. MALDI-TOF mass spectrometry

MALDI-TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) and Post Source Decay (PSD)/MALDI-TOF MS was performed on an Ultraflex workstation (Bruker Daltronics GmbH, Germany). MALDI-TOF MS spectra were recorded in the negative mode using a mixture of galacturonic acid oligosaccharides for calibration. PSD/MALDI-TOF MS was recorded in the positive mode using a mixture of malto-dextrins for calibration. The samples were mixed with a matrix solution (1 μL of sample in 9 μL of matrix). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1 mL mixture of acetonitrile–water (300:700 μL). Of the prepared sample and matrix solution 2 μL was put on a gold plate and dried with warm air.

2.9. HPSEC of colanic acid

Colanic acid (2 mg) was dissolved in 1 mL of distilled water and analysed by high-performance size exclusion chromatography (HPSEC) using three TOSOHAAS TSK-Gel columns in series (4000-, 3000-, 2500-PWXL) preceded by a TSK guard column (40 × 6 mm) according to Verhoef et al.[3]

3. Results and discussion

3.1. Production of colanic acid degrading enzyme

A mixed microbial culture producing colanic acid degrading activity was obtained from an enrichment culture, derived from composted fines blended with chicken manure and wood bark, growing on colanic acid. The enrichment procedure resulted in a mixed culture, and in spite of repeated efforts no single microorganism able to produce activity against colanic acid could be isolated from it. For convenience reasons, the colanic acid degrading enzyme is called colanase in the following sections.

3.2. Purification of colanase

The purification procedure of colanase is summarised in Table 1. The specific activity of the crude enzyme was 113 μU/mg. The active pool after MonoQ anion exchange chromatography showed a threefold increase in specific activity (348 μU/mg) retaining 95% of the initial activity. This purification step was followed by gelfiltration using a prepgrade Superdex 200 column resulting in an active pool with a specific activity of

Table 1. Summary of the colanase purification

Purification step	*V* (mL)	Activity (μU[a]/mL)	Protein (mg/mL)	Specific activity (μU/mg)	Activity yield (%)	Purification factor
Crude enzyme preparation	15.0	57	0.50	113	100	
MonoQ 5/50 GL	13.5	60	0.17	348	95	3
HiLoad Superdex 200 16/60	6.0	41	0.02	1903	30	17

[a] 1 U = 1 μmol of reducing sugars released per minute.

1903 μU/mg, 30% of the enzyme activity was recovered. The apparent molecular weight was found at 775 and 337 kDa suggesting that the enzyme could be present in two different forms. However, upon Native PAGE electrophoresis, as shown later, it turned out that the enzyme could not migrate into the gel indicating that these molecular weight values are based on nonspecific interaction of the protein with the column material resulting in a delayed elution of the column.

3.3. Native PAGE

The colanase preparation was further purified by means of native PAGE electrophoresis, which showed the presence of three major protein bands. In order to determine the position of the active protein band, the crude enzyme preparation was applied to several lanes of a 5–15% Tris/HCL Ready Gel (BioRad). Individual bands were tested using the BCA reducing sugar assay and HPSEC and showed that colanase did not migrate into the stacking gel, which indicates that the enzyme has a very high-molecular weight. SDS-PAGE analysis of the nonmigrated proteins revealed the presence of at least six protein bands (Fig. 2) indicating that the enzyme is incorporated into a large multi protein complex, in which –S–S– bridges probably play an important role. Future work will be directed toward finding out whether one of the subunits present in the protein complex is responsible for the colanase activity or whether the full complex is necessary for degradation.

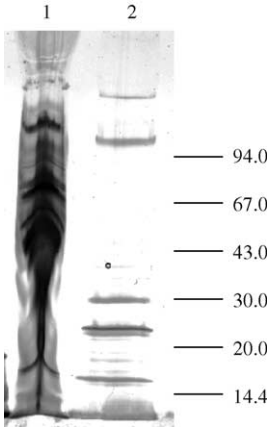

Figure 2. SDS-PAGE gel of isolated colanase isolated from Native PAGE gel; lane 1: crude colanase preparation, lane 2: isolated colanase.

3.4. Characterisation of colanase

The optimum temperature of the enzyme complex was found at 50 °C in 50 mM phosphate buffer pH 7. From the temperature stability tests it could be concluded that the activity of the enzyme started to decrease at temperatures above 40 °C (Fig. 3a). However at 50 °C, still 70% of the activity was left. The pH optimum of the enzyme complex was determined using McIlvaine

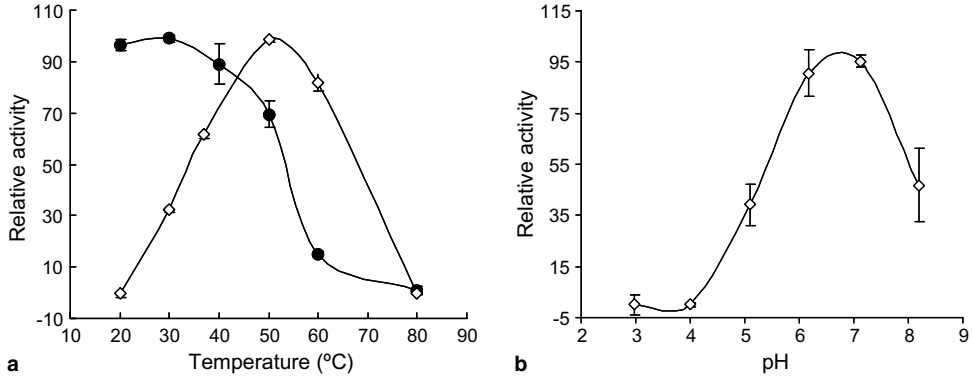

Figure 3. Temperature optimum (–◇–) and stability (–●–) (a) as well as pH optimum (b) of partially purified colanase.

buffers (Fig. 3b). The optimum pH of the enzyme complex was found at pH 7.

3.5. Effect of substrate modification

Colanic acid is *O*-acetylated and contains a pyruvate ketal at the terminal galactose of its side chain, which could be selectively removed by alkaline or acid treatment, respectively. The relative activities of the enzyme towards the native, depyruvylated and de-*O*-acetylated were 1.0:0.3:3.75, respectively.

HPSEC analysis of the different colanase digests showed that even after 48 h (outside the linear rate of degradation) the effect of the removal of pyruvate and *O*-acetyl could clearly be observed by the amount of low-molecular weight material formed (results not shown). Obviously, depyruvylation by autohydrolyses has caused a slight drop in molecular weight of colanic acid.

The substrate specificity of the enzyme was investigated further by determination of the Michaelis Menten constants K_m and V_{max} of the enzyme for the different modified substrates (Fig. 4a/b and Table 2). The removal of the *O*-acetyl moieties by saponification resulted in an increased catalytic activity (V_{max}) of the enzyme by approximately a factor 3, if compared to degradation of the native substrate, while the affinity of the enzyme for the substrate ($1/K_m$) was lowered with a factor ~12. Removal of the pyruvate moiety lowered both the affinity and the catalytic activity of the enzyme by a factor 8 and 2, respectively. The decrease in affinity upon removal of the pyruvate ketals obviously shows that the formation of the enzyme–substrate complex is driven by electrostatic interactions, which is reflected by the fact that below pH 4, no activity of the enzyme complex could be detected (Fig. 3b). Since, *O*-acetyl groups could introduce steric hindrance and therefore shield the negatively charged groups from interaction with the enzyme complex it seems logical that a three-fold increase in maximum velocity compared to degradation of the native substrate was observed upon removal of the *O*-acetyl groups. However, in contrast to this assumption, removal of the *O*-acetyl groups also resulted in a decrease in affinity of the enzyme for the substrate and substrate inhibition (dotted line Fig. 4a) at higher concentrations of native colanic acid was observed.

Following these observations it was concluded that apart from electrostatic interactions, also hydrophobic interaction through the *O*-acetyl groups present plays

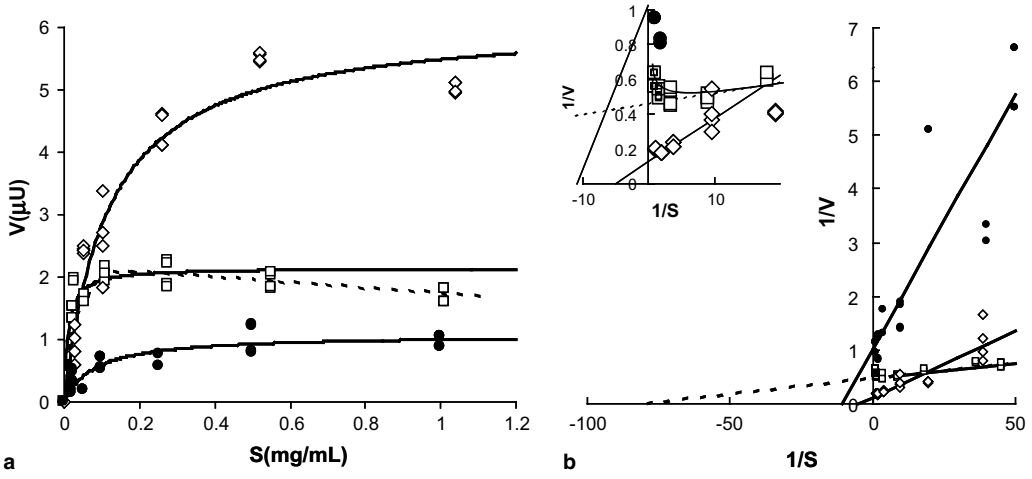

Figure 4. (a) Reaction rate (V) in µU against different concentrations of native, de-*O*-acetylated and depyruvated colanic acid (mg/mL) together with the trend lines ($y = V_{max}x/K_m + x$) used for determination of K_m and V_{max}. The dotted line for native colanic acid shows substrate inhibition at higher concentrations; (b) Lineweaver and Burk plot of the same data ($1/V$ vs $1/S$). (–□–) native; (–◇–) de-*O*-acetylated; (–●–) depyruvated.

Table 2. Extrapolated $1/K_m$ and V_{max} values for incubation of native, de-*O*-acetylated and depyruvated colanic acid with colanase together with the correlation coefficient for the Michaelis–Menten equation regression fit and the same values determined from the Lineweaver and Burk plot

Sample	Michaelis–Menten equation			Lineweaver and Burk plot		
	$1/K_m$ (mg/mL)	V_{max} (µU/min)	R^2	$1/K_m$ (mg/mL)	V_{max} (µU/min)	R^2
Native	100	2.15	0.85	79	2.15	0.79
De-*O*-acetylated	9	6.12	0.94	5	9.87	0.80
Depyruvated	12	1.08	0.82	11	0.91	0.80

Due to substrate inhibition the K_m and V_{max} values for the native substrate should be seen as apparent values.

an important role in the formation of the enzyme–substrate complex. Furthermore, the results indicate that *O*-acetylation of the native substrate gives rise to substrate inhibition probably by the formation of unproductive substrate binding or by strong (hydrophobic) interactions hindering the release of the formed product. However, it cannot be ruled out that this is also an effect of viscosity increase at higher colanic acid concentrations hindering the effective diffusion of the substrate to the enzymes catalytic centre.

3.6. Mode of action

The oligomers formed by the cell free culture and the purified enzyme were analysed by MALDI-TOF MS (Fig. 5). Upon prolonged degradation, the enzyme released oligomers representing the repeating unit of colanic acid with varying degrees of *O*-acetylation. Repeating units without and with 1, 2 or 3 *O*-acetyl groups were found at *m*/*z* 1041, 1083, 1125 and 1167, respectively. The location of these *O*-acetyl groups on the colanic acid repeating units will be discussed later. By comparing the products from the crude enzyme preparation with the purified preparation it became clear that the crude preparation contained fucosidase side activity. This can be deduced from the appearance of the same series of oligomers however with the loss of 1 fucopyranose (−146) in the case of the crude enzyme (Fig. 5a/c). This side activity was removed during purification (Fig. 5b/d).

The linkage cleaved by the enzyme for both native and de-*O*-acetylated colanic acid was determined using Post Source Decay (PSD)/MALDI-TOF MS analysis in the positive mode. Figure 6 shows the spectrum for the de-*O*-acetylated substrate, annotated according to the systematic fragmentation nomenclature of Domon and Costello.[16] PSD analysis mainly resulted in the formation of Y_j and Z_j fragments from the terminal end of

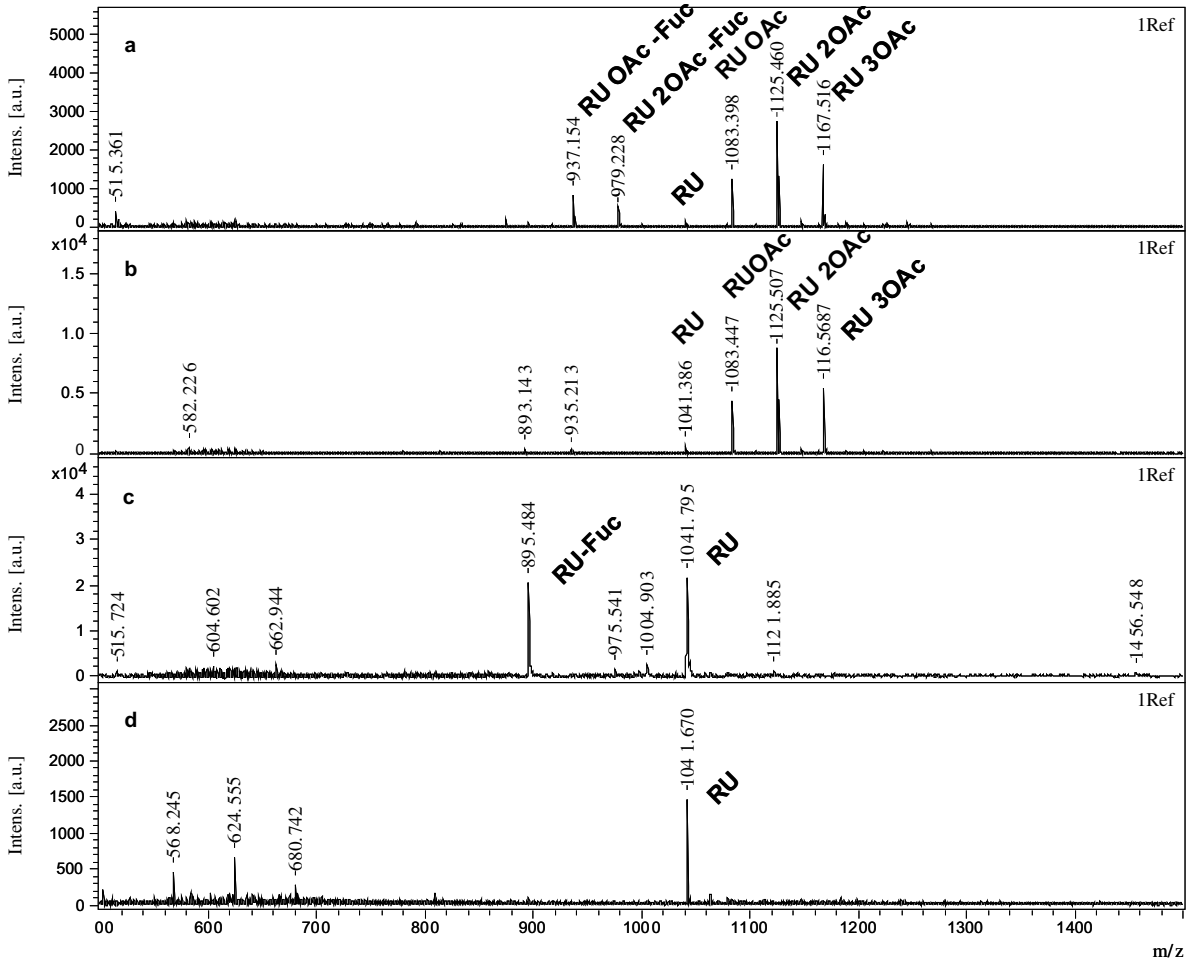

Figure 5. MALDI-TOF MS spectra of both native colanic acid and de-*O*-acetylated colanic acid incubated with crude colanase or purified colanase recorded in the negative mode (M−1): (a) native colanic acid incubated with crude colanase preparation; (b) native colanic acid incubated with purified colanase preparation; (c) de-*O*-acetylated colanic acid incubated with crude colanase preparation; (d) de-*O*-acetylated colanic acid with purified colanase preparation. RU = repeating unit.

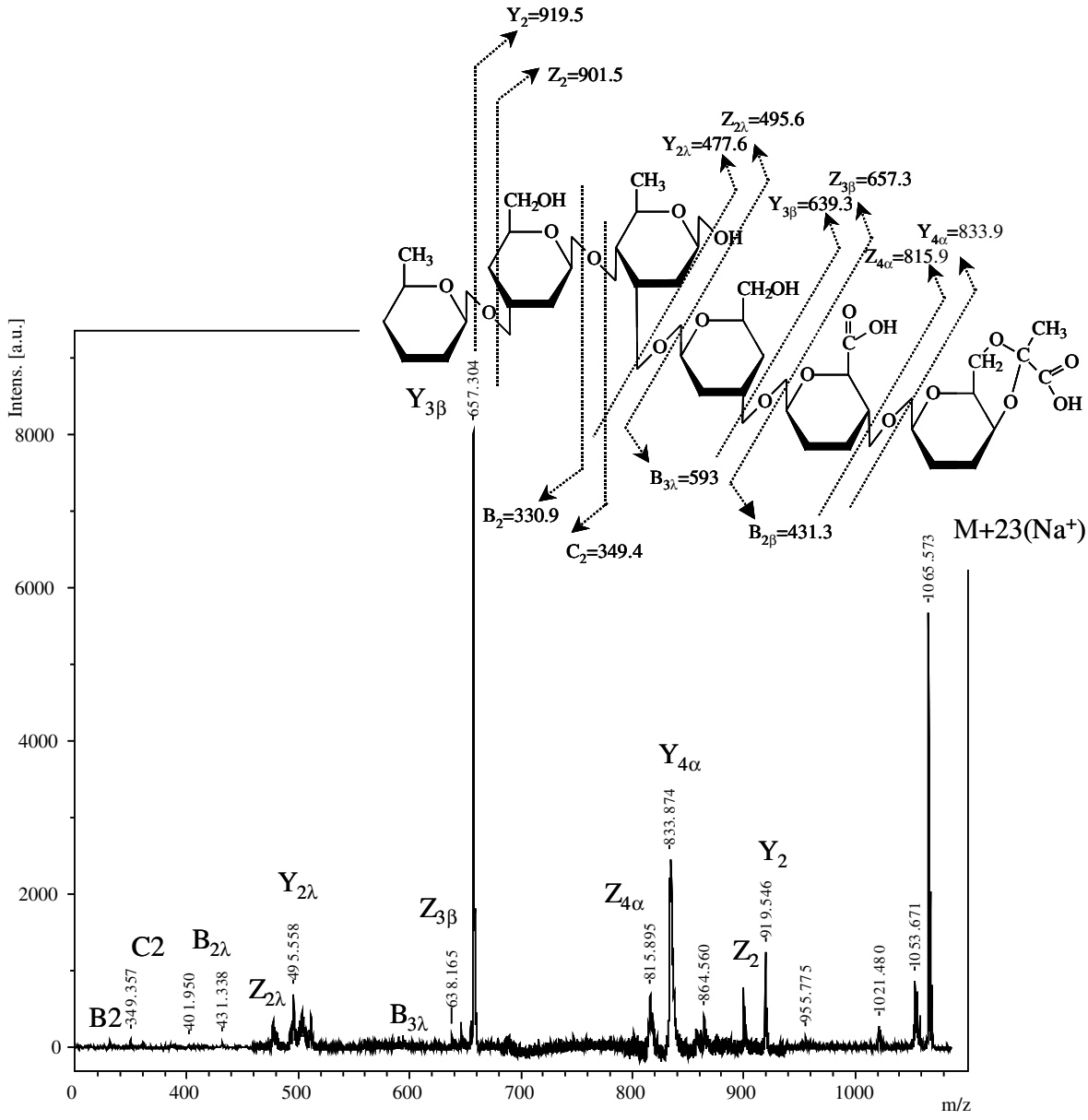

Figure 6. PSD/MALDITOF MS fragmentation pattern of de-*O*-acetylated colanic acid after colanase incubation analysed in the positive mode (M+213 (Na^+)). Peaks are coded with their mass and corresponding ion. Above the spectrum the structure of the repeating unit of colanic acid is given together with the fragmentation pattern. The alcohol groups and protons in the molecular structure are not included so as to make the structure less complex.

the side chain. Additionally the release of fragments Y_2 and Z_2 at *m/z* 919.5 and 901.5, respectively, was observed, representing the loss of the α-L-Fuc*p* residue from the nonreducing end of the core chain of the oligosaccharide. This indicates that the enzyme splits colanic acid between the two adjacent fucopyranosyl residues present in the backbone. The release of fragments B_1, $B_{1\alpha}$, C_1 and $C_{1\alpha}$ was not observed. However, both B_2 and C_2 at *m/z* 330.9 and 349.4, respectively, were present in the spectrum. The latter two fragments represent the release of the →1)-α-L-Fuc*p*-(1→3)-β-D-Glc*p* dimer from the nonreducing end of the branched fucose. This also proves that the enzyme splits between the two adjacent fucopyranosyl residues of the backbone of colanic acid. A similar fragmentation pattern was found for the native EPS after enzyme treatment and PSD MALDI-TOF MS analysis (results not shown). These findings made it possible to classify the novel enzyme as a 1,4-β-fucoside hydrolase.

From the PSD spectra of the native EPS after enzymatic degradation the position of two of the three possible *O*-acetyl groups could be assigned. Fragment Y_2 was found after the loss of the fucose residues with as well as without *O*-acetyl, confirming the *O*-acetylation

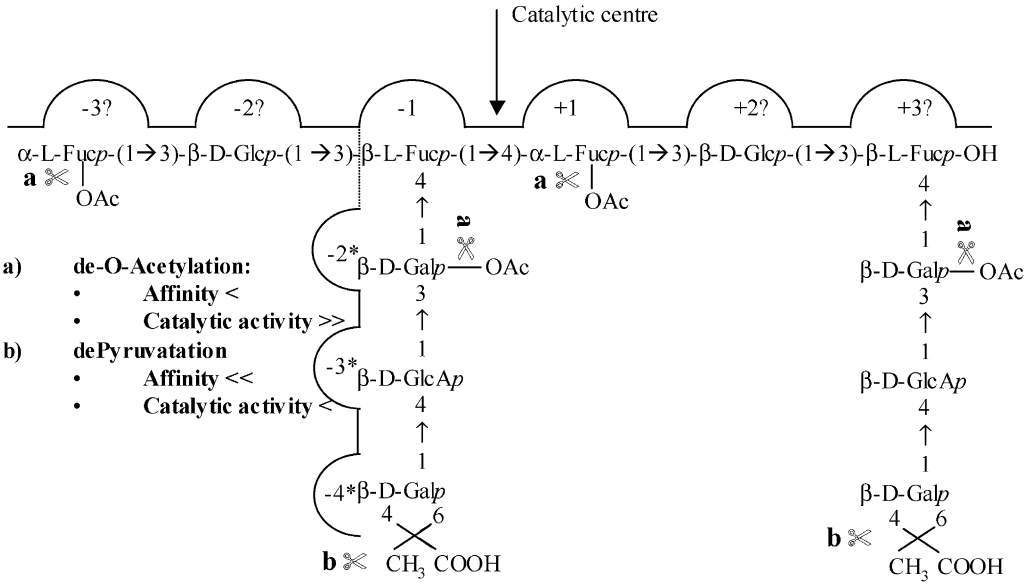

Figure 7. Putative representation of the different subsites of colanase and the effects of colanic acid modification upon the enzymes affinity and catalytic activity. −2*, −3* and −4* are side-chain specific subsites.

site found before by Garegg and co-workers.[9,10] Furthermore, fragment $Y_{2\lambda}$ represents the loss of the trimeric side chain with an *O*-acetyl group attached to the (1→3)-linked β-Gal*p* residue. The position of the third possible *O*-acetyl group could not be established. These findings showed that the nonbranched fucose, formerly assumed to be the only acetylation site based upon resistance to β-elimination of this (1→4)-linked α-L-Fuc*p* residue,[9,10] is not the only *O*-acetylation site of colanic acid.

Combining the results from the present study made it possible to postulate a putative subsite model for the enzyme. This model is shown in Figure 7 and summarises some cleavage characteristics of the enzyme. Although subsite models are normally only used for linear polysaccharide backbones, the results obtained show that in this case the side chain plays an important role in the affinity of the substrate for the enzyme and that a more complex subsite model is necessary. Therefore, unconventionally, the side chain is incorporated within the subsite model showing the terminal pyruvated galactose of the side chain at subsite −4* and the *O*-acetylated fucose in the backbone at subsite +1. No clear evidence for the position of subsites −2, −3, +2 and +3 was obtained leaving the assignment of these subsites on a putative base.

4. Conclusions

Purification of colanase resulted in the recognition of a novel 1,4-β-fucoside hydrolase. The enzyme could be used in a broad pH and temperature range and therefore could make it applicable to prevent slime formation in, for example, paper machines.[17–19]

The fucoside hydrolase purified was an *endo* acting, hydrolytic enzyme cleaving colanic acid between the two adjacent fucopyranosyl residues present in the backbone of colanic acid resulting in the formation of single repeating units with varying degrees of *O*-acetylation. Kinetic studies and substrate modifications revealed that the affinity of the enzyme for colanic acid is based upon electrostatic and hydrophobic interactions and that removal of the *O*-acetyl moieties results in an increase in catalytic activity.

The enzyme in this study was different from the ones described earlier, because it showed a far more significant drop in molecular weights of the substrate after incubation than the one described by van Speybroeck et al.,[7] which only showed a molecular weight decrease of approximately 2000–400 kDa, instead of complete release of single hexameric repeating units of about 1 kDa after exhaustive incubation with the present enzyme. The enzyme described by Sutherland[11] was phage assisted and different to the enzyme characterised in this work, which was found to be far more effective in the release of repeating units. Furthermore, the novel enzyme characterised was found to be a true fucoside hydrolase being able to degrade linkages between two fucopyranose moieties instead of the linkage between fucose and glucose as in the case of the enzyme described by Sutherland.[11]

The novel 1,4-β-fucoside hydrolase has a high-molecular weight and apparently is a part of a multiprotein complex consisting of at least six different proteins with varying molecular weight. Future work will be directed towards the determination of partial amino acid sequences of the individual proteins for PCR cloning of the respective genes.

Acknowledgements

We wish to thank Ing. Margaret Bosveld at Wageningen University, Laboratory of Food Chemistry, for the very useful discussions and help with purifying the enzyme. Also we would like to thank Dr. Reinhard Wilting at Novozymes A/S (Denmark) for his advise with respect to the possible cloning of the enzyme described.

The work described has been carried out with financial support from the Commission of the European Communities, specific RTD programme 'Competitive and Sustainable Growth', G1RD-CT2000-00387, 'Eco-efficient novel enzymatic concepts for slime control in pulp and paper processing (Slimezymes)'.

It does not necessarily reflect the Commission's views and in no way anticipates the Commission's future policy in this area.

References

1. Wingender, J.; Neu, R. T.; Flemming, H.-C. What are Bacterial Extracellular Polymeric Substances? In *Microbial Extracellular Polymeric Substances; Characterisation, Structure and Function*; Wingender, J., Neu, R. T., Flemming, H.-C., Eds.; Springer: Heidelberg, 1999, pp 1–19.
2. Safade, T. *Paper Technol. Ind.* **1988**, 280–285.
3. Verhoef, R.; Schols, H. A.; Blanco, A.; Siika-aho, M.; Rättö, M.; Buchert, J.; Lennon, G.; Voragen, A. G. J. *Biotechnol. Bioeng.* **2005**, *91*, 91–105.
4. Grant, W. D.; Sutherland, I. W.; Wilkinson, J. F. *J. Bacteriol.* **1969**, *100*, 1187–1193.
5. Goebel, W. F. *Proc. Nat. Acad. Wash.* **1963**, 49464.
6. Danese, P. N.; Pratt, L. A.; Kolter, R. *J. Bacteriol.* **2000**, *182*, 3593–3596.
7. Speybroeck, M. M. van; Bruggeman, G.; Poele, J. van; Pee, K. L. I. van; Vandamme, E. J. WO96/31610 1996.
8. Sutherland, I. W. *J. Gen. Microbiol.* **1969**, *57*, 18.
9. Garegg, P. J.; Lindberg, B.; Onn, T.; Sutherland, I. W. *Acta Chem. Scand.* **1971**, *25*, 2103–2108.
10. Garegg, J.; Lindberg, B.; Onn, T.; Holme, T. *Acta Chem. Scand.* **1971**, *25*, 1185–1194.
11. Sutherland, I. W. *Eur. J. Biochem./FEBS* **1971**, *23*, 582–587.
12. Rättö, M.; Verhoef, R.; Blanco, A.; Suihko, M.-L.; Schols, H. A.; Voragen, A. G. J.; Siika Aho, M. *J. Ind. Microbiol. Biotechnol.*, submitted for publication.
13. Meeuwsen, P. J. A.; Vincken, J.-P.; Beldman, G.; Voragen, A. G. J. *J. Biosci. Bioeng.* **2000**, *89*, 107–109.
14. Bradford, M. M. *Anal. Biochem.* **1976**, 72248–72254.
15. Verhoef, R.; Waard, P. de; Schols, H. A.; Siika-aho, M.; Voragen, A. G. J. *Carbohydr. Res.* **2003**, *338*, 1851–1859.
16. Domon, B.; Costello, E. C. *Glycoconjugate J.* **1988**, 5397–5409.
17. Väisänen, O.; Nurmiaho-Lassila, E.; Marmo, S.; Salkinoja-Salonen, M. *Appl. Environ. Microbiol.* **1994**, *60*, 641–653.
18. Klahre, J.; Flemming, H. C. *Water Res.* **2000**, *34*, 3657–3665.
19. Edwards, J. C. *Tappi J.* **1996**, *79*, 71–77.