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Nitrile Hydratase and Amidase from *Rhodococcus rhodochrous* Hydrolyze Acrylic Fibers and Granular Polyacrylonitriles

M. M. TAUBER,[1,2] A. CAVACO-PAULO,[2] K.-H. ROBRA,[1] AND G. M. GÜBITZ[1]*

Institut für Mikrobiologie und Abfalltechnologie, Technische Universität Graz, A-8010 Graz, Austria,[1] *and Departamento de Engenharia Textil, Universidade do Minho, P-4800 Guimaraes, Portugal*[2]

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Rhodococcus rhodochrous **NCIMB 11216 produced nitrile hydratase (320 nkat mg of protein^{-1}) and amidase activity (38.4 nkat mg of protein^{-1}) when grown on a medium containing propionitrile. These enzymes were able to hydrolyze nitrile groups of both granular polyacrylonitriles (PAN) and acrylic fibers. Nitrile groups of PAN40 (molecular mass, 40 kDa) and PAN190 (molecular mass, 190 kDa) were converted into the corresponding carbonic acids to 1.8 and 1.0%, respectively. In contrast, surfacial nitrile groups of acrylic fibers were only converted to the corresponding amides. X-ray photoelectron spectroscopy analysis showed that 16% of the surfacial nitrile groups were hydrolyzed by the *R. rhodochrous* enzymes. Due to the enzymatic modification, the acrylic fibers became more hydrophilic and thus, adsorption of dyes was enhanced. This was indicated by a 15% increase in the staining level (*K/S* value) for C.I. Basic Blue 9.**

The ability to degrade nitriles is quite common among microorganisms. The potential of nitrile-degrading enzymes for biotransformations, waste treatment, and the production of herbicide-resistant plants has been assessed (14, 29). Stereoselective hydrolysis of nitriles and amides with whole cells or isolated enzymes has been reported for a number of strains, such as *Pseudomonas putida* (6, 28), *Rhodococcus erythropolis* (10), *Rhodococcus equi* (19), and *Rhodococcus rhodochrous* (13, 21). In Japan, enzymes from *Pseudomonas chlororaphis* and *R. rhodochrous* are used for the production of low-cost chemicals, such as acrylamide (23).

In nature, three different groups of enzymes are involved in the microbial hydrolysis of nitriles. Nitrilases (EC 3.5.5.1 and 3.5.5.7) hydrolyze nitriles to the corresponding carboxylic acids, forming ammonia; nitrile hydratases (EC 4.2.1.84) form amides from nitriles which can be subsequently hydrolyzed by amidases (EC 3.5.1.4). The nocardiaform actinomycete *R. rhodochrous* has been reported to produce both the nitrilase and the nitrile hydratase/amidase system, depending on the inducer used (32).

Various nitrilases that hydrolyze aromatic and aliphatic substrates have been described for *R. rhodochrous*. Some of these enzymes were selectively induced with benzonitrile and propionitrile, respectively (11). Nitrilases from *R. rhodochrous* have been used for the production of acrylic and methacrylic acid (23). "Aliphatic" nitrilases, such as those from various *R. rhodochrous* strains (NCIMB 11216, K22, and J1), seem to be quite unusual among microorganisms (11, 15, 23). Formerly, nitrilases have been thought exclusively to hydrolyze aromatic substances while aliphatic nitriles have been believed to be degraded by a nitrile hydratase/amidase enzyme system (15).

The reaction mechanism, regulation, and photoactivation of nitrile hydratases, which usually consist of α and β subunits containing either nonheme iron or cobalt atoms, have been studied in detail (14, 27). *R. rhodochrous* has been reported to produce a high-molecular-weight nitrile hydratase and a low-molecular-weight nitrile hydratase, which were selectively induced by the reaction products (amides) and by urea, respectively (16, 22, 32). The high-molecular-weight nitrile hydratase from *R. rhodochrous* is used for industrial production (30,000 tons/year) of acrylamide (14, 26, 32).

In this paper, we report on the modification of polyacrylonitrile (PAN) fibers and granulates using enzymes from *R. rhodochrous* NCIMB 11216, which has previously been used for chemoselective hydrolysis of nitriles at our university (13). Although acrylonitrile seems to be one of most suitable substrates for both nitrile hydratase (25) and nitrilase from *R. rhodochrous* (15), there are no reports in the literature on the enzymatic hydrolysis of PANs.

PAN fibers have a slightly increasing share of 2,700 tons/year in 1997, holding approximately 10% of the global synthetic fiber market. Several attempts have been made in the last few years to increase the ecoefficiency of the production processes, including the subsequent dyeing of the fibers. However, various chemical methods for the modification of PAN fibers to make them more hydrophilic and thereby enhance dye uptake have not been very successful. Partial hydrolysis of surfacial nitrile groups into amides and acids, which resist strong acids and alkali, leads to irreversible yellowing of the fabrics. Additionally, elevated reaction temperatures, aggressive chemicals, and higher concentrations of dimethyl sulfoxide (12) would lead to unwanted changes in the macroscopic behavior of the fibers. Long-term mild alkali treatment led to lower dye uptake, which was explained by hydrolysis of ester groups of the copolymers to less hydrophilic hydroxyl (-OH) groups. Thus, selective enzymatic hydrolysis of surfacial nitrile groups of PAN fibers offers a promising alternative to chemical processes. The utilization of endoglucanases to enhance the properties of cellulosic fibers has been studied extensively, including contributions from our laboratories (2, 3, 9, 18), and several cellulase-based processes have been introduced into the textile industry. Similarly, nitrile-degrading enzymes could have an immense potential in this area.

MATERIALS AND METHODS

Organism and culture conditions. *R. rhodochrous* NCIMB 11216 was inoculated from agar stock cultures on potato dextrose agar and grown in three steps using two rich media (media 1 and 2) and a minimal medium containing propionitrile as described previously (13). Additionally, a trace element solution containing the following (concentrations in milligrams per liter are in parenthe-

* Corresponding author. Mailing address: Institut für Mikrobiologie und Abfalltechnologie, Technische Universität Graz, Petersgasse 12, A-8010 Graz, Austria. Phone: 43 316 8738312. Fax: 43 316 8738815. E-mail: guebitz@ima.tu-graz.ac.at.

ses) was added: $ZnSO_4 \cdot 7H_2O$ (100), $MnCl_2 \cdot 4H_2O$ (30), H_3BO_3 (300), $CoCl_2 \cdot 6H_2O$ (200), $CuSO_4 \cdot 5H_2O$ (10), $NiCl_2 \cdot 6H_2O$ (20), and $NaMoO_4 \cdot 2H_2O$ (30). Cultures were grown for 24 h in each medium at 30°C and 180 rpm. A quarter of the cell harvest was used for inoculation of medium 2, and the total cell harvest was used for inoculation of the minimal medium. Harvesting of cells was performed at 2,500 × *g* for 10 min at 4°C. Cells were disrupted by ultrasonic treatment on ice and centrifuged again to obtain the cell-free supernatant (enzyme preparation).

Enzyme assay. The incubation mixture for the determination of nitrile-degrading enzyme activity contained 40 mg of the enzyme preparation (protein) per liter, 25 mM acrylonitrile or acrylamide, and 20 mM K_2HPO_4/KH_2PO_4 as a buffer (pH 6.5). The mixture was incubated at 30°C and 200 rpm, and samples were taken every 5 min. The reaction was stopped after 60 min by thermal inactivation of the enzymes, which were subsequently removed by centrifugation. Gas chromatography was used for analysis of the reaction products. The measurement was carried out using an HP5890 SerII gas chromatograph equipped with an HP-Innowax cross-linked polyethylene glycol column (30 m by 0.25 mm). The column temperature was raised to 220°C in accordance with the following profile: 0 to 1 min, 120°C; 1 to 11 min, increase to 220°C; 11 to 13 min, 220 °C.

Protein assay. Protein adsorbed to the fabric was measured by the Lowry method using bovine serum albumin as the standard (8). A 150-mg fabric sample was incubated with 5 ml of a solution containing (grams per liter) 16.6 Na_2CO_3, 4.0 NaOH, 0.2 Na-K tartrate, and 0.1 $CuSO_45H_2O$ while shaking for 10 min at 22°C. Thereafter, 10% (vol/vol) Folin Ciocalteau phenol reagent (1 N; Merck) was added and the A_{750} was read after 30 min of incubation at 22°C. The protein content in solution was determined by the Bradford method (1). Specific enzyme activities were calculated based on the protein content of the enzyme preparation.

Enzymatic treatment of PAN. Two granular PAN standards (40 and 190 kDa; PSS Polymer Standards Service GmbH, Mainz, Germany) were treated with the crude enzyme preparation. The incubation mixture contained 1% (wt/vol) PAN and 0.36 or 0.0036% (wt/vol) enzyme preparation (protein) in 1 ml of 57 mM phosphate buffer (pH 7.0). Experiments were carried out in 1.5-ml screw-cap plastic flasks, which were shaken at 300 rpm at 25°C for 72 h. The reaction was stopped by centrifugation, and ammonia that had been released into the supernatant was monitored as described previously during incubation for 15 min at 50°C (4).

Enzymatic treatment of acrylic fibers. Commercial acrylic fibers consisting of PAN and 7% (wt/wt) vinyl acetate as a copolymer were obtained from Fisipe SA, Lavradio, Portugal. The acrylic fibers were washed twice with tap water at 60°C for 1 h each time. Enzymatic treatment was performed in screw-cap plastic flasks (100 ml) at 30°C on a Linitest machine (horizontal shaker commonly used for treatment of fabrics) rotating at 30 rpm for 3 days. The incubation mixture contained 1.0 g of acrylic fibers, 5.0 mg of enzyme preparation (protein) or heat-inactivated enzymes (control), and 0.5% (vol/vol) dimethyl formamide (DMF) in 40 ml of 57 mM phosphate buffer set to different pH values as indicated below. Subsequently, the fabrics were washed with 1% (wt/vol) Na_2CO_3 at pH 11 for 30 s, rinsed with distilled water for 10 s, and dried for 1 h at 60°C. The release of acetic acid was monitored using an enzyme-based assay kit from Boehringer.

Dyeing experiments. Dyeing of enzymatically treated fabrics was carried out using the Ahiba Spectradye system from Datacolor International (Lucerne, Switzerland). A 2.0-g fabric sample, 80 mg of methylene blue (C.I. 52015 Basic Blue 9; Sigma), and 20 mg of Coomassie brilliant blue G (C.I. 42655 Acid Blue 90; Sigma) were incubated in 40 ml of distilled water (bath ratio, 1:20) at 50°C and 60 rpm for 60 min (temperature increase to 40°C from 0 to 5 min, addition of dye from 5 to 10 min at 40°C, increase to 50°C from 10 to 20 min, 50°C from 20 to 80 min, and cooling down to 20°C from 80 to 85 min). Dye uptake was quantified by measuring the *K*/*S* value, which correlates to the reflectance (*R* in percent) in the following way:

$$K/S = \left\{ \left[1 - \left(\frac{R}{100} \right) \right]^2 / (2R) \right\} \cdot 100$$

K/*S* values of the fabrics were measured using an ACS reflectance spectrometer.

XPS. X-ray photoelectron spectroscopy (XPS) analysis was carried out on an ESCALAB 200A (VG Scientific, West Sussex, United Kingdom). The X-ray tube had Mg Kα radiation, and the analyzing mode was CAE. The following parameters were set for region spectra: maximum count rate, 29,905/s; analyze, 20 eV; step size, 0.10 eV; dwell time, 200 ms; number of channels, 201; number of scans, 10; time for region, 402 s.

If not otherwise stated, all experiments were carried out in duplicate using analytical-grade chemicals from Merck.

RESULTS

Production of nitrile-degrading enzymes. *R. rhodochrous* NCIMB 11216 produced nitrile-degrading enzymes (nitrilase and/or the nitrile hydratase/amidase enzyme system), as indicated by the formation of propionic acid when the bacteria were grown on culture medium containing propionitrile. During cultivation (24 h), 10% of the propionitrile present was transformed into propionic acid.

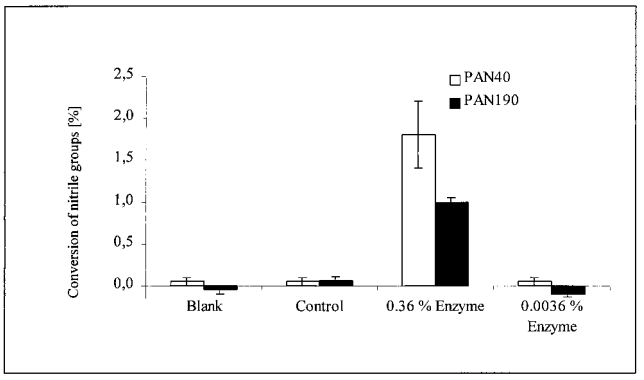

FIG. 1. Hydrolysis of granular PANs with *R. rhodochrous* enzymes at different concentrations after 72 h of incubation (expressed as a percentage of total possible conversion; 0.36 and 0.0036 g of enzyme g of PAN^{-1}; control, 0.36 g of heat-inactivated enzyme g of PAN^{-1}).

On acrylonitrile as the biocatalytic substrate, a nitrile hydratase activity of 14.2 nkat mg^{-1} (cell dry weight) was measured in the cell extract, corresponding to a specific activity of 320 nkat mg^{-1} (based on the measured protein content of the enzyme preparation). Using acrylamide as a substrate for the enzyme the *R. rhodochrous* enzyme preparation showed amidase activities of 1.7 nkat mg^{-1} (cell dry weight) and 38.4 nkat mg^{-1} (protein), respectively.

Enzymatic treatment of PANs. The *R. rhodochrous* enzymes were able to hydrolyze nitrile groups of granular PAN, as indicated by the release of ammonia. Less ammonia was released from a high-molecular-mass PAN (190 kDa; PAN190) (Fig. 1).

Surprisingly, no release of ammonia could be detected when acrylic fabrics were treated with the *R. rhodochrous* enzyme preparation. In addition, no acetic acid could be detected although the enzyme preparation was able to hydrolyze ethyl acetate and naphthyl acetate (data not shown). However, increases in the efficiency of subsequent dyeing of 37 and 81% based on *K*/*S* values were measured using methylene blue (C.I. Basic Blue 9) and Coomassie brilliant blue G (C.I. Acid Blue 90), respectively, compared to a control with heat-inactivated enzymes (Fig. 2). These results indicated that surfacial nitrile groups were hydrolyzed to the corresponding amides. The basic dye methylene blue interacts both with the carbonyl groups

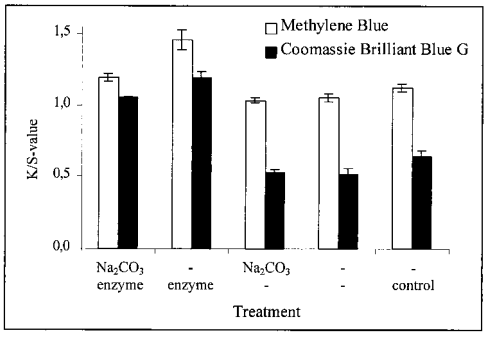

FIG. 2. Dyeing of enzymatically pretreated acrylic fibers with methylene blue and Coomassie brilliant blue G and effect of posttreatment with Na_2CO_3 (control, heat-inactivated enzyme).

TABLE 1. Influence of enzymatic hydrolysis of acrylic fibers at different pHs and DMF and protein concentrations on dyeing with methylene blue

Enzyme loading on acrylic fibers					
1.0% DMF, 1.0 mg of enzyme g of acrylic fiber^{-1}		pH 6.5, 1.0 mg of enzyme g of acrylic fiber^{-1}		pH 6.5, 0.5% DMF	
pH	$\Delta K/S^b$	DMF [% v/v]	$\Delta K/S^b$	Enzyme concn (mg g^{-1})	$\Delta K/S$
6.0	0.111	0.0	0.063	1.0	0.133
6.5	**0.124**	0.5	**0.133**	5.0	**0.156**
7.0	0.065	1.0	0.124		
		1.5	0.030		

[a] Experimental settings corresponding to values in boldface were used in subsequent optimization steps (from left to right).
[b] An increase in the *K/S* value, which correlates with reflectance, indicates increased dye uptake (see Materials and Methods).

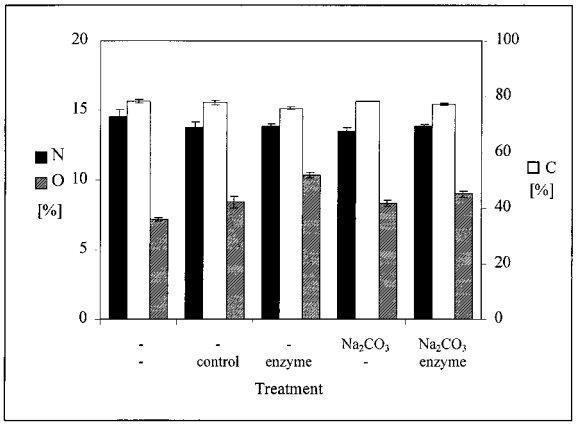

FIG. 3. Changes in the elementary composition of enzymatically treated acrylic fibers (determined by XPS analysis; error bars indicate values from two separate experiments).

from the copolymer vinyl acetate and with amides formed enzymatically from PAN. The acid dye Coomassie brilliant blue G interacts with its sulfonylic groups with the protonated nitrogen of enzymatically formed amide groups.

The treatment was performed at different pH values. The best results were obtained at pH 6.5 (Table 1). Addition of DMF to increase the accessibility of nitrile groups at a concentration no higher than 0.5% (vol/vol) was found to be beneficial for the treatment. An enzyme concentration of 5 mg g^{-1} did not show any substantial increase in the *K/S* value compared to 1 mg g^{-1}; however, this concentration was used in all further experiments to detect any potential leveling effect (Table 1).

Measurements of the protein concentration of the incubation mixture and of the protein on the washed fabrics revealed that 4.4 of the 5.0 mg g of protein^{-1} added had adsorbed to the fibers. Ninety-seven percent of the adsorbed protein could be removed by posttreatment with Na_2CO_3. The posttreatment reduced the beneficial effect of the enzyme treatment from a 37 to a 15% *K/S* increase for methylene blue, while the enzyme effect was more pronounced when dyeing with Coomassie brilliant blue G was followed by posttreatment (100% increase in the *K/S* value; Fig. 2). Removal of the adsorbed protein was necessary to avoid nonleveling of the fabrics due to adsorption of the dye on the protein. This effect was less pronounced for Coomassie brilliant blue G.

To confirm our hypothesis that the increase in dyeing efficiency resulted from the formation of surfacial amide groups, we compared several techniques for the quantification of the enzyme effect on acrylic fibers. Both the Fourier-transform infrared and RAMAN microspectroscopy methods failed for this purpose. XPS is a surface analysis technique allowing measurement of the sample composition to a depth of 5 nm. Using this technique, we were able to detect the changes caused by the enzyme treatment.

Measuring the elementary composition of acrylic fibers with XPS, we found that the oxygen content increased significantly in the enzyme-treated samples. There was a 45% increase between the blank and enzyme-treated samples. Compared to a control with heat-inactivated enzyme, a 27% increase can be ascribed to the effect of the active enzymes. The enzyme effect was less pronounced when PAN was posttreated with Na_2CO_3, which could be due to alkaline hydrolysis of the nitrile groups. The carbon content of all enzyme-treated samples decreased slightly in response to the increase in oxygen, while the nitrogen content showed only insignificant variations (Fig. 3). These results indicate that nitrile groups were converted into amides. About 16% (5% for Na_2CO_3-posttreated samples) of the nitrile groups in enzymatically treated PAN fibers were converted to amides. However, the actual values reveal the elementary composition of the fiber surface and not that of the whole sample (Fig. 4), which is in agreement with the substantial increases in dyeing efficiency caused by the enzyme treatment.

DISCUSSION

R. rhodochrous NCIMB 11216 can transform a wide range of nitriles (11, 13). In this paper, we report for the first time that the enzymes of *R. rhodochrous* can also hydrolyze surfacial nitrile groups of PAN fibers and granulates. *R. rhodochrous* hydrolyzed monomeric acrylonitrile and acrylamide with nitrile hydratase and amidase, respectively, when grown in the presence of propionitrile. Recently, a nitrilase from *R. rhodochrous* NCIMB 11216 hydrolyzing aliphatic nitriles has been described which was produced during cultivation of the organism in the presence of propionitrile and benzonitrile (11). Previously, other authors reported the existence of both a nitrile hydratase/amidase system and a nitrilase in *R. rhodochrous* NCIMB 11216 (13).

On acrylonitrile, the nitrile hydratase activity of the *R. rhodochrous* enzyme preparation was 14.2 nkat mg^{-1} (cell dry

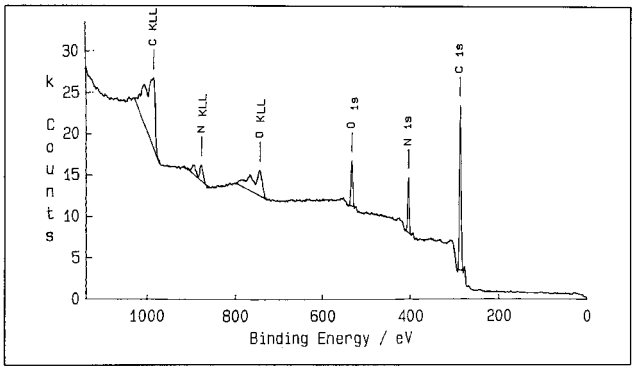

FIG. 4. XPS analysis of acrylic fibers. A typical peak survey diagram is shown. KLL peaks are due to the Auger effect.

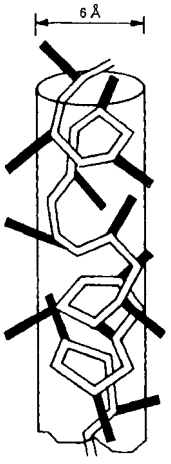

FIG. 5. Model of PAN structure. Reprinted with permission from the original publisher (7).

weight). Previously, on the same substrate, nitrilase activities of 11 and 63 nkat mg^{-1} were measured for *R. rhodochrous* NCIMB 11216 and J1, respectively, when these strains had been cultivated in the presence of caprolactam (24). However, this "nitrilase" activity on acrylonitrile could also result from the cooperative action of nitrile hydratase and amidase because the intermediately formed amides cannot be detected if instantly hydrolyzed by amidase. In this case, the presence of nitrile hydratase can be determined if amidase is selectively inhibited (17). We found that the nitrile hydratase activity of *R. rhodochrous* NCIMB was significantly higher than amidase activity (1.7 nkat mg^{-1} on acrylamide), which is in contrast to observations for enzymes from *R. erythropolis* (17).

Hydrolysis of nitrile groups of PANs with enzymes from *R. rhodochrous* was studied with two granular PAN standards (40 and 190 kDa) and commercial acrylic fibers containing vinyl acetate as a copolymer. Interestingly, nitrile groups of both PAN40 and PAN190 were partially converted to the corresponding acid while nitrile groups of acrylic fibers were only hydrolyzed to the amide. Thus, nitrile hydratases hydrolyzed surfacial nitrile groups of acrylic fibers but the resulting amides were obviously not accessible to amidases. In agreement with these results, amidases from *R. rhodochrous* NCIMB 11216 and AJ270 have been reported to be generally more sensitive to the geometry of the substrate than nitrile hydratases (5, 20). Esterases from *R. rhodochrous* have previously caused problems during biotransformations of nitriles with ester function (13). Nevertheless, we have not observed any concurrent hydrolysis of vinyl acetate by esterases during hydrolysis of surfacial nitrile groups of acrylic fibers.

Pretreatment of acrylic fibers with *R. rhodochrous* enzymes improved fabric-dyeing efficiency, as indicated by *K*/*S* value increases. However, in the case of methylene blue, posttreatment of the fabrics with sodium carbonate to remove adsorbed enzymes seemed to be necessary to gain leveling. Nonleveling during dyeing is caused by adsorption of dye to the fiber-bound enzymes. This phenomenon depends both on the type of enzyme and on the dyeing conditions, as we have previously shown for indigo backstaining during cellulose washing (3).

Surfacial nitrile groups of acrylic fibers were hydrolyzed by the *R. rhodochrous* enzymes to a maximum of only 16%, although acrylonitrile is known to be a very good substrate for both nitrilases and nitrile hydratases from these organism (15, 25). However, the enzyme reaction on PAN is restricted by several factors related to the properties of the polymer. The structure of PAN is assumed to have a rigid, irregularly helical conformation of the polymer chain including planar zigzag packing (Fig. 5). The cylinders, with a diameter of 6 nm, can bind to each other through the antiparallel orientation of the side groups. In the model of Warner, fibers are composed of fibrillar subunits containing distinct regions of amorphous and partially ordered material (30).

Enzyme adsorption to and desorption from the polymer affect the hydrolysis rate. Additionally, the crystallinity and hydrophobicity of PAN fibers may limit the accessibility of nitrile groups to the enzyme. Similarly, the structure and properties of cellulose influence its enzymatic degradation. It has been suggested that endoglucanases randomly cleave cellulose into smaller fragments, generating new ends, which are then hydrolyzed endwise by the action of cellobiohydrolases. These latter enzymes are also thought to erode crystalline regions of cellulose, making them more susceptible to endoglucanase attack (31).

In contrast to that of synthetic PAN polymers, enzymatic hydrolysis of natural polymers like cellulose has been investigated for decades. The architecture of cellulose-degrading enzymes has been studied in detail, and the function of the active sites and binding domains related to specificities for cellulose has been elucidated. Based on this knowledge, these enzymes and genetically improved products have been successfully applied in the textile industry. Similarly, nitrile-degrading enzymes could have an immense potential for the improvement of PAN fibers. Thus, future investigations could focus on the reaction mechanisms of nitrilases and nitrile hydratases in relation to the structure and properties of PANs.

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