ADVANCES IN BIOCHEMICAL ENGINEERING

Volume 5

Editors T. K Ghose, A. Fiechter, N. Blakebrough

Managing Editor A Fiechter

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（内部交流）

With 31 Figures

Springer-Verlag
Berlin · Heidelberg · New York 1977

生物化学工程进展　第5卷

本卷主要叙述了真菌水解酶在工业生产上的应用，并对这种酶的作用机制及生产中遇到的问题做了详细的评价，认为真菌水解酶较为有前途，利用纤维素制造可食用葡萄糖对解决人类粮食问题提供了前景。可供研究酶、生化设计、大专院校工作人员参考。

目次如下：①真菌纤维素（水解）酶的生产，②纤维物质酶解的评价，③在热钝化时植物性微生物核酸之破坏，④在哺乳动物异物代谢研究中，细胞和微生物模型的应用，⑤发酵罐中"混合作用"的特性描述，⑥整体细胞的固定化。

第1—2卷已编入 F30/51 及 F51/102。

ISBN 3-540-08074-0 Springer-Verlag Berlin · Heidelberg · New York
ISBN 0-387-08074-0 Springer-Verlag New York · Heidelberg · Berlin

Library of Congress Catalog Card Number 72-152360.
Printed in Germany.

Typesetting, printing, and bookbinding: Brühlsche Universitätsdruckerei Gießen.

Editorial Guidelines

The aim of this series is to keep bioengineers and microbiologists informed of the fundaments and advances pertaining to the biochemical processes they need for the construction of bio-plants—be they for water purification, obtaining enzymes or antibiotics, for breeding yeasts, or those required for other special biochemical or biosynthetic operations. This series will likewise familiarize the biochemist with how the engineer thinks and proceeds in his work, as well as with the constructive aids at his disposal. Providing the various specialists with such extensive information is not an easy task: the backgrounds of the biochemist, the microbiologist, and the engineer are founded on entirely different bases; yet they must work side-by-side in the constantly changing field of biochemical engineering.

With this as foremost consideration, the Editors will make a special effort to present a selection of premises as well as new findings and ways of applying innovations that arise. The field of biochemical engineering is still developing and making advancements in highly industrialized nations; it is also becoming increasingly significant in those lands plagued by food shortages, which are still wrestling with problems of development today. Of primary interest for these countries are discoveries of methods for obtaining valuable natural substances and for disposing of wastes—where possible, recycling them into useful and even highly beneficial products. Advances in Biochemical Engineering can provide them with relevant contributions dealing with means of supplying food—proteins, in particular. Therefore, just as biochemistry and technology are brought together in this series, the reader will be offered contributions from industrial nations and from those countries that are presently in need of progress in the area of technology.

The Editors look forward to a strong influx of manuscripts and will do their utmost to insure the series' rapid publication. They will be published in English in order to afford the widest possbile outreach. Editors and Editorial Board are now prepared to accept manuscripts for consideration.

The Editors

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Production of Cellulolytic Enzymes by Fungi

T.-M. ENARI and P. MARKKANEN
Technical Research Centre of Finland, Biotechnical Laboratory,
Box 192, SF-00121 Helsinki 12, Finland

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Summary

Microorganisms able to utilize cellulose are found amongst bacteria, actinomycetes, and higher fungi. *Trichoderma viride* seems to be the best presently available organism for the production of extracellular cellulases. Most studies concerning the nature and mode of action of cellulases have also been carried out using this organism.

The enzymatic degradation of cellulose is a complex process requiring the participation of at least three types of cellulolytic activity: exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-glucosidase. In the hydrolysis of native cellulose exo- and endoglucanase act synergistically to produce cellobiose, which is then degraded to glucose by β-glucosidase. Some other enzymes may also be involved in the process, but definite evidence of this is lacking.

The synthesis of cellulase in *T. viride* is controlled by a repressor-inducer mechanism. The production of cellulases is thus greatly influenced by the carbon source in the medium. Glucose or other rapidly metabolized compounds cannot be used. Cellulose or some cellulosic material is probably the most suitable substrate for industrial cellulase production.

Cellulases are produced by surface culture methods, but the high price of the enzymes precludes their use in the biotechnical process industry. Research work aimed at developing industrial cellulase production by submerged fermentation has reached the pilot plant stage. The production is not economical at present, but continued research into improvement of microbial strains and process optimization may solve the problems in the near future.

1. Introduction

The food and energy shortages in the world have directed the interest of applied research workers toward the search for hitherto unused renewable resources. Cellulose is the major constituent of all plant material. It forms about one third of the woody tissues and is constantly replenished by photosynthesis. It is thus natural that a growing interest should be shown in the application of cellulolytic microbes and their enzymes to the utilization of cellulosic materials. The main use of extracellular cellulolytic enzymes would be in the hydrolysis of cellulosic materials in order to produce fermentable sugars for various biotechnical processes. One other important use would be in the treatment of fodder grain to increase its digestibility. Furthermore, cellulolytic organisms which do not excrete cellulases could be utilized for the production of single-cell protein by direct cultivation on cellulosic materials. A prerequisite for all technical applications of this kind is a thorough knowledge of the enzymes and their properties as well as the development of industrial processes for cellulase production.

Microorganisms producing enzymes hydrolyzing β-glucosidic linkages are widely distributed amongst various taxa. The ability to utilize cellulose is found amongst bacteria, actinomycetes, and higher fungi. The concept of cellulases can be limited to enzymes capable of degrading highly ordered cellulose into sugars small enough to pass through the microbial cell wall. It has been found that the degradation of cellulose is a complex process accomplished by the synergistic action of several enzymes. This review is confined to the enzyme complex hydrolyzing native cellulose to fermentable sugars. Since fungi are the organisms most likely to be used for industrial production of cellulases only fungal enzymes are discussed.

2. Nature of Cellulases

2.1 Cellulolytic Enzymes

The degradation of crystalline cellulose is a complex process, requiring the participation of many enzymes. It is now well established that there are at least three different types of cellulolytic activity: exo-β-1,4-glucanase (E. C. 3.2.1.–), endo-β-1,4-glucanase (E. C. 3.2.1.4), and β-glucosidase (E. C. 3.2.1.21). A strong synergistic effect has been observed between exo- and endoglucanases hydrolyzing crystalline cellulose (Avicel), but not when hydrolyzing acid-swollen cellulose [1]. β-Glucosidases hydrolyze cellobiose and short-chain cello-oligosaccharides to glucose, but have no effect on cellulose. Some β-glucosidases attack aryl-β-glucosides, but not cellobiose [2].

The first hypothesis concerning the nature of enzymatic hydrolysis of cellulose was put forward by Reese *et al.* [3]. They reported the existence of a nonhydrolytic enzyme, C_1, which initiated the hydrolysis of native cellulose by breaking hydrogen bonds between cellulose chains. This first step was a prerequisite for hydrolysis by hydrolytic enzymes,

C_x [4]. It was also believed that those microorganisms unable to grow on native cellulose did not synthesize C_1-enzyme. This model for the hydrolysis has subsequently been extensively questioned. In particular, the nature of the initial step in the hydrolysis of native cellulose is still obscure. At present the most generally accepted view is that C_1-enzyme is an exo-β-1,4-glucanase. In the case of *Trichoderma viride* and *T. koningii* purified C_1 has been shown to be a cellobiohydrolase [5–13]. Clearly we should now reconsider the theory of Reese and coworkers and redefine the mechanisms of cellulase action in the light of new understanding of the properties of cellulases. According to the present view, cooperative action of endo- and exoglucanases hydrolyzes crystalline cellulose to soluble cello-oligosaccharides, mainly cellobiose, which is released by exo-glucanases [1, 11, 12, 14].

2.1.1 Exo- and Endoglucanases

Wood and McCrae [14, 15] separated the cellulase complex produced by *Trichoderma koningii* into eight pure components using gel filtration, ion exchange chromatography and isoelectric focusing. These components were a single exo-β-1,4-glucanase, C_1, five endo-β-1,4-glucanases, C_x, and two β-glucosidases. The complex thus contains many isoenzymes. The same authors reported that the exoglucanase was splitting off cellobiose from the non-reducing end of the cellulose chain [8, 11]. Thus, it may be systematically designated β-1,4-glucan cellobiohydrolase (E.C.3.2.1.–). The endoglucanases hydrolyze β-1,4-glucans in a random fashion and are systematically called β-1,4-glucan glucanohydrolases (E.C.3.2.1.4). The endoglucanases can be differentiated by the randomness of their attack on carboxymethyl cellulose (CMC) and by the rate of solubilization of phosphoric acid-swollen cellulose [8, 11].

Eriksson and coworkers studied the cellulase system of the rot fungus *Sporotrichum pulverulentum* (formerly called *Chrysosporium lignorum*) [1, 16]. Eriksson and Pettersson isolated five endo-β-1,4-glucanases and an exo-β-1,4-glucanase [16]. After isolation these proteins were found to be pure when tested using various methods. The same authors also quantitatively determined the ratio of activities between these five components to be 4 : 1 : 1 : 1 : 1.

Pettersson [12] fractionated the cellulase complex of *T. viride* into four components using gel chromatography, ion exchange chromatography, biospecific chromatography, and isoelectric focusing. Two of the components were endoglucanases, one was an exoglucanase, and one a cellobiase. The exoglucanase was shown to be a cellobiohydrolase, which was inhibited by cellobiose. Consequently β-glucosidase greatly accelerates the action of exoglucanase on microcrystalline cellulose by removing cellobiose.

It therefore seems clear that fungi produce at least five different endo-β-1,4-glucanases, the old C_x-components, varying in degree of randomness of hydrolytic action. So far only one exo-β-1,4-glucanase has been purified and fully characterized. It has, however, been shown clearly that all known organisms hydrolyzing native cellulose are able to produce at least one exo-β-glucanase. In the case of *T. viride, T. koningii* and *S. pulverulentum*, this enzyme is β-1,4-glucan cellobiohydrolase.

It has also been claimed that fungi produce a β-1,4-glucan glucosylhydrolase, but none of these enzymes has been isolated in a pure state. Preparations releasing glucose from

cellulosic substrates have been isolated from culture media of *T. viride* [17] and *Aspergillus niger* [18]. However, these preparations were not pure enough to completely exclude the presence of cellobiase.

2.1.2 β-Glucosidases

The third activity involved in the breakdown of cellulose is β-glucosidase or cellobiase (E.C.3.2.1.21), which hydrolyzes mainly cellobiose, but also higher cellodextrins to glucose. These enzymes accelerate the hydrolysis of crystalline cellulose by removing cellobiose, which is an inhibitor of exo-β-glucanase. β-glucosidases are widespread in fungi. Bucht and Eriksson [2] isolated both β-glucosidase and aryl-β-glucosidase from *Stereum sanguinolentum*. *T. koningii* produces two β-glucosidases [11].

2.2 Properties of Cellulases

The molecular weights of the five endoglucanases isolated from *Sporotrichum pulverulentum* vary between 28300 and 37500 [16]. Small differences in the amino-acid composition have also been found. The isoelectric points vary between 4.20 and 5.32, making possible their separation by isoelectric focusing. With the exception of one component, all endoglucanases are glycoproteins. In Table 1 some properties of cellulases isolated from *T. viride* are summarized according to Pettersson [12]. The molecular weights of the exo- and endoglucanases of *T. viride, T. koningii, Fusarium solani* and *Penicillium funiculosum* lie in the region 40000 ... 75000, with the exception of the low-molecular-weight components from *T. koningii* and *T. viride*. These have a molecular weight of 12500 ... 13000 [12, 14].

Table 1. Some properties of cellulolytic enzymes isolated from *Trichoderma viride* [12]

Type of enzyme	Molecular weight	Isoelectric point	Carbohydrate content (per cent)	Activity toward different substrates			
				CMC	Microcrystalline cellulose	Reprecipitated cellulose	Cellotetraose
Exo-β-1,4-glucanase	42000	3.79	9	–	+	+	+
Endo-β-1,4-glucanase I	12500	4.60	21	+	–	+	+
Endo-β-1,4-glucanase II	50000	3.39	12	+	–	+	+
β-Glucosidase	47000	5.74	0	–	–	–	+

Thermostability is one of the most important technical properties of cellulases, since the hydrolysis of cellulose proceeds faster at higher temperatures. Endoglucanases are more stable than exoglucanases. Endoglucanases are quite stable for up to 4 hrs at 60° C and pH 5.0. β-Glucosidase and exoglucanase of *T. koningii* resemble one another in their

heat stability at 60° C: they loose about 80% of their original activity at 60° C and pH 5.0 in 4 hrs [14]. In the presence of cotton the cellulases of *T. koningii* and *F. solani* are remarkably stable, showing no loss of activity when incubated for 4 weeks at 37° C and pH 5.0 [14].

2.3 Mode of Action of Cellulases

Wood and McCrae purified the exoglucanase (C_1-component) of *Trichoderma koningii* using ion exchange chromatography on a DEAE-Sephadex column and pH gradient elution [8, 14, 19]. The low-molecular-weight endoglucanase, the removal of which does not affect the kinetics of solubilization of cotton fiber [20], was first separated from the culture filtrate by gel chromatography on a Sephadex G-75 column. The remaining fraction containing endoglucanases (C_x) and β-glucosidases, was separated according to the scheme in Fig. 1.

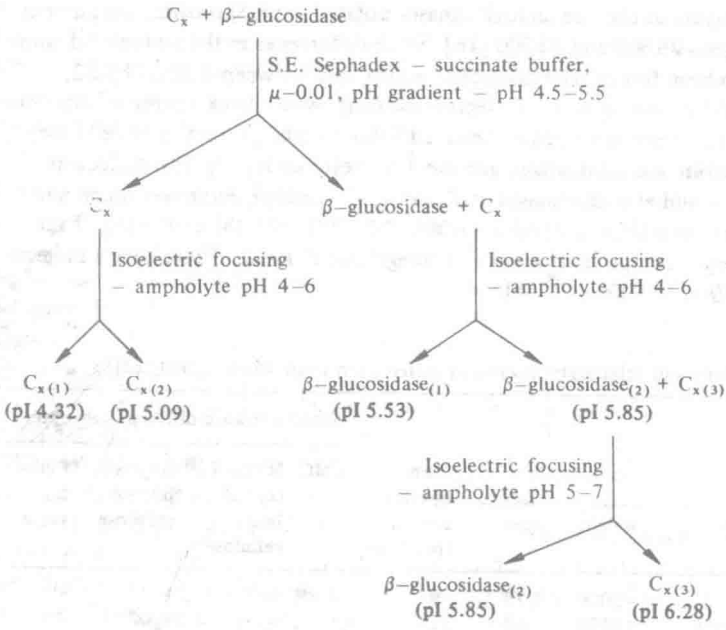

Fig. 1. Wood's and McCrae's scheme for fractionation of cellulolytic enzymes from *Trichoderma koningii* [11]

The synergistic properties of the separated enzymes were studied by the same authors [11, 14]. They could reconstitute the cellulase complex from the fraction because the recoveries of the enzymes and protein were very high, over 90% [11]. Table 2 shows some of the reconstitution results.

Table 2. Relative cellulase activities of the components of *Trichoderma koningii* cellulase alone and in combination [11]

Enzyme	Relative cellulase activity (%)
C_1	< 1
$C_{x(1)}$	< 1
$C_{x(2)}$	< 1
β-Glucosidase$_{(1)}$	0
β-Glucosidase$_{(2)}$	0
$C_1 + C_{x(1)} + C_{x(2)}$	24
$C_1 + \beta$-glucosidase$_{(1+2)}$	5
$C_1 + C_{x(1+2)} + \beta$-glucosidase$_{(1+2)}$	103
20–80% sat. $(NH_4)_2SO_4$ fraction	100

All of the original cellulase activity was reconstituted when all the components, $C_1 + C_{x(1+2)} + \beta$-glucosidase$_{(1+2)}$, were recombined in their original proportions. The most potent synergistic effect was found between exoglucanase (C_1) and the endoglucanase component ($C_{x(2)}$) when cotton was used as substrate. $C_{x(1)}$ and C_1 act synergistically on cellulose, but the low-molecular-weight C_x-component showed no synergistic effect at all. Glucose and cellobiose were the main products when exo- and endoglucanases were combined. However, the proportion of glucose was low: 8% when the combination $C_1 + C_{x(1)}$ was used, and 14% for $C_1 + C_{x(2)}$.

Wood also observed other differences in the hydrolytic capacities of *T. koningii* cellulases. The endoglucanase, $C_{x(1)}$-component, hydrolyzed 29% of phosphoric-acid-swollen cellulose in 4 h, while the endoglucanase, $C_{x(2)}$, hydrolyzed 83%, and exoglucanase, C_1, 32% [11]. The endoglucanase, $C_{x(2)}$-component, is more random in its action than $C_{x(1)}$. Obviously, therefore, the combination of exoglucanase with the endoglucanase, $C_{x(2)}$-component, hydrolyzes cotton cellulose more efficiently; endoglucanase, $C_{x(2)}$, opens more end groups for the action of exoglucanase. This finding supports the theory that endoglucanases initiate the attack on native cellulose.

Exo-β-glucanase from *S. pulverulentum* showed no viscosity-decreasing activity toward CMC [13]. The main product, cellobiose, is released in the α-configuration [1]. Eriksson and Pettersson [16] found that the weight ratio of exoglucanase protein to endoglucanase protein was 1 : 1. They also found a strong synergistic action between exo- and endoglucanases when hydrolyzing crystalline cellulose, but not when hydrolyzing phosphoric-acid-swollen cellulose [1]. Endoglucanase pretreatment also increased the production of cellobiose from cotton cellulose by exoglucanase. This also supports the theory that the endoglucanases open chain ends for exoglucanases.

This evidence strongly supports the mechanism for enzymatic degradation of cellulose as described by Pettersson [12] (Table 3).

In the first reaction free ends for exoglucanase are released at the sites of non-crystalline regions of the cellulose. This kind of mechanism was first suggested by Eriksson [21, 22]. Different endoglucanases have different substrate specificities and therefore can attack a variety of substrates.

Table 3. A mechanism for enzymatic cellulose degradation [12]

1. Native cellulose	$\xrightarrow{\text{Endoglucanase}}$	Cellulose[a]
2. Cellulose[a]	$\xrightarrow{\text{Exoglucanase}}$	Cellobiose
3. Cellobiose	$\xrightarrow{\beta\text{-glucosidase}}$	2 Glucose

[a] Formed from native cellulose by the action of the endoglucanase on non-crystalline regions of the cellulose fiber. Free chain ends are created.

The initiation mechanism of the degradation of cellulose has not yet been completely clarified. It is still possible that hitherto unknown enzymes are involved in the degradation. The existence of one such enzyme was demonstrated by Eriksson and coworkers [23]. The quantitative purification of both exo- and endoglucanases from culture filtrate of *S. pulverulentum* made it possible for them to reconstitute the culture solution using purified enzymes.

The reconstituted solution contained the same quantities of endo- and exoglucanases as the original culture solution. The concentrated unfractionated culture solution degraded 52.1% of de-waxed cotton, whereas the reconstituted solution degraded only 20% [13, 23]. They believed that an additional enzyme important for the degradation of crystalline cellulose was present in the culture solution, but not in the reconstituted solution. When the culture solution was incubated with nitrogen instead of air, the degree of cellulose degradation decreased from the original 52.1% to 21.5% (Table 4). This indicates that there is an additional oxidizing enzyme involved in the degradation of cellulose. It was also shown that the same enzyme is present in the culture solutions of other cellulolytic fungi (Table 5). Eriksson has suggested that the probable mode of action of this oxidizing enzyme comprises insertion of uronic-acid moieties into the cellulose, thus breaking the hydrogen bonds between chains. However, the enzyme has not yet been characterized and purified in sufficient amounts for a final verification of this.

Table 4. Degradation of cotton cellulose by enzymes from *Sporotrichum pulverulentum* [23]

Tube No.	Enzyme preparation	Cellulose degradation, weight loss %
1	Concentrated culture solution	52.1 (oxygen atmosphere)
2	Concentrated culture solution	21.5 (nitrogen atmosphere)
3	Mixture of endo- and exoglucanases	20.0
4	Endo-β-1,4-glucanases	0.0
5	Exo-β-1,4-glucanase	0.0

Most of the studies concerning the degradation of cellulose have been made using pure cellulose as substrate. In natural materials cellulose is usually present as a complex. *S. pulverulentum* produces the enzyme cellobiose: quinone oxidoreductase which participates in the degradation of cellulose in wood [13, 24, 25]. This enzyme needs a quinone as a cosubstrate (quinones are released from lignin) and therefore cannot function in the degradation of pure cellulose. Thus, degradation studies with pure cellulose as substrate may be misleading.

Table 5. Degradation of cotton cellulose by cell-free, concentrated culture solutions of four different cellulose-degradating fungi in presence and absence of oxygen [23]

Organism	Cellulose degradation (weight loss %)	
	O_2-atmosphere	N_2-atmosphere
Sporotrichum pulverulentum[a]	52.1	21.5
Polyporus adustus[b]	42.6	18.0
Myrothecium verrucaria[b]	33.6	17.0
Trichoderma viride[c]	20.0	10.0

[a] Culture solution concentrated 50 times.
[b] Culture solution concentrated 30 times.
[c] Culture solution concentrated 20 times.

It is apparent, therefore, that the degradation of cellulose is a complicated process. It has been clearly demonstrated that there is a synergistic effect between exo- and endo-glucanases. Furthermore, β-glucosidase is needed for the removal of cellobiose, which otherwise inhibits the action of exoglucanase. The oxidizing enzyme observed by Eriksson [13] may be involved in the degradation of crystalline cellulose, but its function has yet to be demonstrated. It is also likely that new kinds of cellulolytic enzymes will be discovered in the near future, as there is a great amount of research activity in this field. It is tempting to replace the old C_1–C_x concept by more precise names, such as exo-β-glucanase and endo-β-glucanase. However, confusion may arise through replacement of the term C_1 by exoglucanase, since there are also exoglucanases which do not attack insoluble cellulose [26]. The mechanisms of the action of the cellulase complex may also be very different in different organisms [27].

2.4 Activity Determinations

Determination of the activities of cellulolytic enzymes is complicated by two factors: 1. In most cases determinations are not made on purified enzymes, but rather on solutions containing a mixture of different cellulolytic enzymes. Because of the synergistic action of these enzymes, the activity measured is greatly influenced by the proportions of different enzymes, which may vary; 2. The substrates used are natural macromolecules, which makes standardization difficult. The ideal substrate would be of low molecular weight and specific. Unfortunately, only in the case of β-glucosidase such a substrate is available.

In developing methods for activity determinations, two different approaches can and have been adopted. In the technical approach the starting point is the use of the cellulases. The main technical use of cellulases is to produce glucose from various cellulosic materials. Hence, this approach leads to a method in which the substrate is a suitable cellulosic material (e.g. filter paper) and the end-product formed, glucose, is measured. Methods of this type give a value for the overall cellulolytic activity, but give no indication of which enzyme is rate-limiting. They are useful for determining the capacity of a certain

enzyme complex to hydrolyze cellulose, but they are not methods for the determination of the activity of individual enzymes.

In the biochemical approach the aim is to determine the activity of individual enzymes. These methods are necessary for research into the biochemical mechanism of enzymatic cellulose degradation. They are also very useful in screening cellulase-producing organisms and in developing enzyme production processes. Thus, measures can be taken to improve the limiting activity. The difficulty in developing methods for individual activities is that it is necessary to know which enzymes are involved in cellulose degradation. Thus, a considerable amount of biochemical research is necessary in the development of such methods. Another difficulty is the lack of specific substrates or inhibitors which would permit measurement of one activity in the presence of other synergistic activities.

2.4.1 Overall Cellulolytic Activity

In determinations of the overall cellulolytic activity, the substrate must resemble the one which will be used in a technical hydrolysis process, i.e. it must be an insoluble cellulosic material which is not too easily hydrolyzed. It must, nevertheless, be a material which can be standardized. Another important factor is the reaction time. Since the substrate is an insoluble fibrous material, time is required for the enzyme to diffuse into the fiber and for the hydrolysis products to diffuse out of the fiber. Another difficulty is caused by the varying accessibility of glucosidic bonds in different regions of the fiber. In order to give a meaningful result, the assay requires a reaction time long enough for hydrolysis of an appreciable fraction of the less accessible bonds. Thus, for overall cellulolytic activity the generally accepted rule of measuring the initial reaction rate cannot be followed.

Various substrates have been proposed. Cotton fiber is one of the most resistant. Avicel, a microcrystalline cellulose, is also difficult to hydrolyze. Suiphite pulps, such as Solka Floc and filter paper, have also been used. Filter paper has proved to be a satisfactory substrate for the measurement of overall cellulolytic activity. The method of Mandels and Weber [28] has gained general acceptance for this purpose. In this method the reducing sugars formed under standard conditions are estimated. The reaction time used is relatively short, one hour, and hence the measurement is based on limited action of the enzymes on the most susceptible regions of the substrate. Increasing the enzyme or substrate concentration leads to increased sugar production, as does a prolonged reaction time. The increase in glucose formation is not linear because the most reactive substrate is converted at the beginning of the reaction period [29]. The activity values are erratic at high glucose concentrations and tend to become less meaningful with highly active preparations. Linearity can be increased by diluting the enzyme, increasing substrate concentration or decreasing the reaction time [30]. It has been suggested that the most reliable quantitative activity determinations ought to involve enzyme units based on the same degree of hydrolysis of the filter paper, e.g. a dilution giving 2 mg of glucose [29] under the test conditions.

The most difficult step in the technical hydrolysis of cellulosic materials is the solubilization of fibrous substrates. It is therefore natural that special emphasis be placed on determination of the solubilizing activity, which has often been referred to as C_1-activity.

It has been shown that solubilization is caused by the synergistic activity of exo-β-glucanase and endo-β-glucanase [30]. Methods for determination of the solubilizing activity make use of cotton fibers, microcrystalline cellulose (Avicel), or hydrocellulose as substrate, with measurement of the production of reducing sugars [28], the loss of weight [31] or the decrease in optical density of a cellulose suspension [32].
When the formation of reducing sugars is measured, the acitivity determined is the sum of different cellulolytic activities, and the result depends on the relative proportions of the different enzymes. The formation of cellobiose or glucose as the end-product depends on the β-glucosidase activity, which can thus greatly influence the result [33]. Determination of the nonsolubilized substrate after enzymic digestion gives a reliable result, but the method is laborious and unsuitable for long series of determinations. Measurements based on the reduction in optical density of cellulose suspensions are useful for the screening of cellulase-producing organisms or mutants. In a plate assay, cellulase-producing organisms can be detected by formation of a clear zone when growing on a medium containing phosphoric-acid-swollen cellulose [34, 35].
The release of dye from a dyed insoluble substrate is a convenient way of measuring the solubilizing activity. Dyed filter paper [36], dyed Solka Floc [37], and dyed Avicel [37] have been used as substrates. There is also a commercial substrate available, Cellulose azure (Calbiochem, Switzerland). The best substrate for determining the solubilizing activity is dyed Avicel SF [33, 37]. Dyed Solka Floc and the commercial Cellulose azure are too easily solubilized. The method using dyed Avicel is convenient and rapid. It is thus a good tool for research into the production of cellulases and screening of cellulolytic microorganisms.
A number of methods making use of less well-defined activities have also been proposed. Such methods include swelling of cotton or paper, maceration of paper, decrease in breaking strength of yarn, thread, or fabrics, and microfragmentation of cellulose micelles [28].

2.4.2 Endo-β-Glucanase

Endo-β-1,4-glucanases randomly attack β-1,4-linkages in CMC or swollen cellulose. The best substrate for the measurement of endo-β-glucanase activity is a soluble cellulose derivative such as CMC. This substrate has been employed by many workers, who measured either the decrease in viscosity [38] or the production of reducing sugars [28]. Measurement of the decrease in viscosity is a very sensitive technique, since even a few breaks in a chain cause a marked decrease in the average chain length. Measurement of the reducing sugars is less sensitive and is also influenced by the presence of other cellulolytic enzymes, especially β-glucosidase. CMC is not, as such, attacked by cellulolytic enzymes other than endo-β-glucanase [12] (Table 1), but the cellobiose formed is, of course, hydrolyzed by β-glucosidase. Ionic-substituted celluloses, for example CMC, are not the ideal substrates for viscometric assays. Difficulties are caused by the fact that the viscosity of ionic substrates is dependent on pH, ionic strength, and polyvalent cations. For this reason, non-ionic-substituted celluloses, such as hydroxyethyl cellulose (HEC), are preferred for the determination of low endo-β-glucanase activities [39].