POSSIBILITIES FOR RECYCLING CELLULASES AFTER USE IN COTTON PROCESSING

By

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Eu gostaria de dedicar este trabalho aos meus queridos pais, Isabel e Fernando, e à minha irmã Isabel.

I would like to dedicate this work to my dear parents, Isabel and Fernando, and to my sister Isabel.

ABSTRACT

Repeated treatments on cotton fabrics, using a total cellulase from *Trichoderma reesei*, showed that active enzymes remained in the treatment liquor and adsorbed on the fabric substrates after five treatment cycles. The activity (measured as fabric weight loss) of the cellulases remaining in solution, however, decreased by 80% over the five cycles. It was suggested that this could be due to end-product inhibition, thermal and or mechanical deactivation, or to the loss of some components of the cellulase complex by preferential and irreversible adsorption to the cotton substrates.

End-product inhibition studies showed that the build-up of cellobiose and glucose in solution would cause less than 25% activity loss after five treatment cycles.

Agitation levels similar to those used in textile processing did not cause significant cellulase deactivation, but incubation of total cellulase solutions for 5h at 50 °C (equivalent to five treatment cycles) reduced their activity by about 20%.

Analysis of cellulase solutions, by fast protein liquid chromatography, before and after adsorption onto cotton, suggested that the cellobiohydrolase II content of the cellulase complex was reduced, relative to the other components, by preferential adsorption. This would lead to a marked reduction in activity in the supernatant after several treatment cycles and top-up with pure cellobiohydrolase II would be necessary unless this component is easily recoverable from the treated fabric. Although desorption studies showed that much of the adsorbed cellulase protein was recoverable by washing in buffer solution, concentration of the washings by ultrafiltration needs to be carefully costed.

Experiments with pure endoglucanases, with and without their cellulose binding domains, suggested that desired cotton finishing effects might be achievable using only cellulase components that are adsorbed reversibly. These would be ideal for recycling.

Ultrafiltration was used successfully to reduce end-product concentrations in treatment liquors, but this technique failed to separate dyes from cellulase proteins. Since dye-cellulase complexes cause backstaining problems, it seems unlikely that cellulases used for enzymatic stonewashing (currently the most important cellulase-finishing process) can be recycled unless a solution to this problem is found. Using only reversibly adsorbed cellulase components may be beneficial also in this context.

Cost estimates suggested that when more than 100 to 125 tonnes per annum of cellulosic fabric are being treated with cellulases, the savings made by recycling enzymes would exceed the capital and running costs of the ultrafiltration unit required for their recovery and concentration.

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LIST OF ABBREVIATIONS

Bis-Tris	2,2-Bis-(hydroxyethyl)-(iminotris)-(hydroxymethyl) methane
BOD	Biochemical Oxygen Demand
BMCC	Bacterial Microcrystalline Cellulose
BSA	Bovine Serum Albumin
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolase
CBM	Carbohydrate Binding Module
C. fimi	*Cellulomonas fimi*
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
DP	Degree of Polymerisation
DSC	Differential Scanning Calorimetry
E	Enzyme
EC	Enzyme Commission
EG	Endoglucanase

ELISA	Enzyme-Linked Immunosorbent Assay
FP	Filter Paper
FPLC	Fast Protein Liquid Chromatography
G_1	Glucose
G_2	Cellobiose
HEC	Hydroxyethylcellulose
HPLC	High Performance Liquid Chromatography
H. insolens	*Humicola insolens*
IUB	International Union of Biochemistry
LAS	Linear Alkyl Benzene Sulfonates
MWCO	Molecular Weight Cut-Off
pI	Isoelectric Point
S	Substrate
TC	Total Crude or Total Cellulase
T. fusca	*Thermomonospora fusca*
T. reesei	*Trichoderma reesei*

UF	Ultrafiltration
UV	Ultraviolet

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AUTHOR'S DECLARATION

The work described in this thesis was carried out in the Department of Textile Engineering at the University of Minho while the author was registered as a part-time research student at De Montfort University.

The work has not been submitted for any other degree and is original work of the author except where acknowledged by reference.

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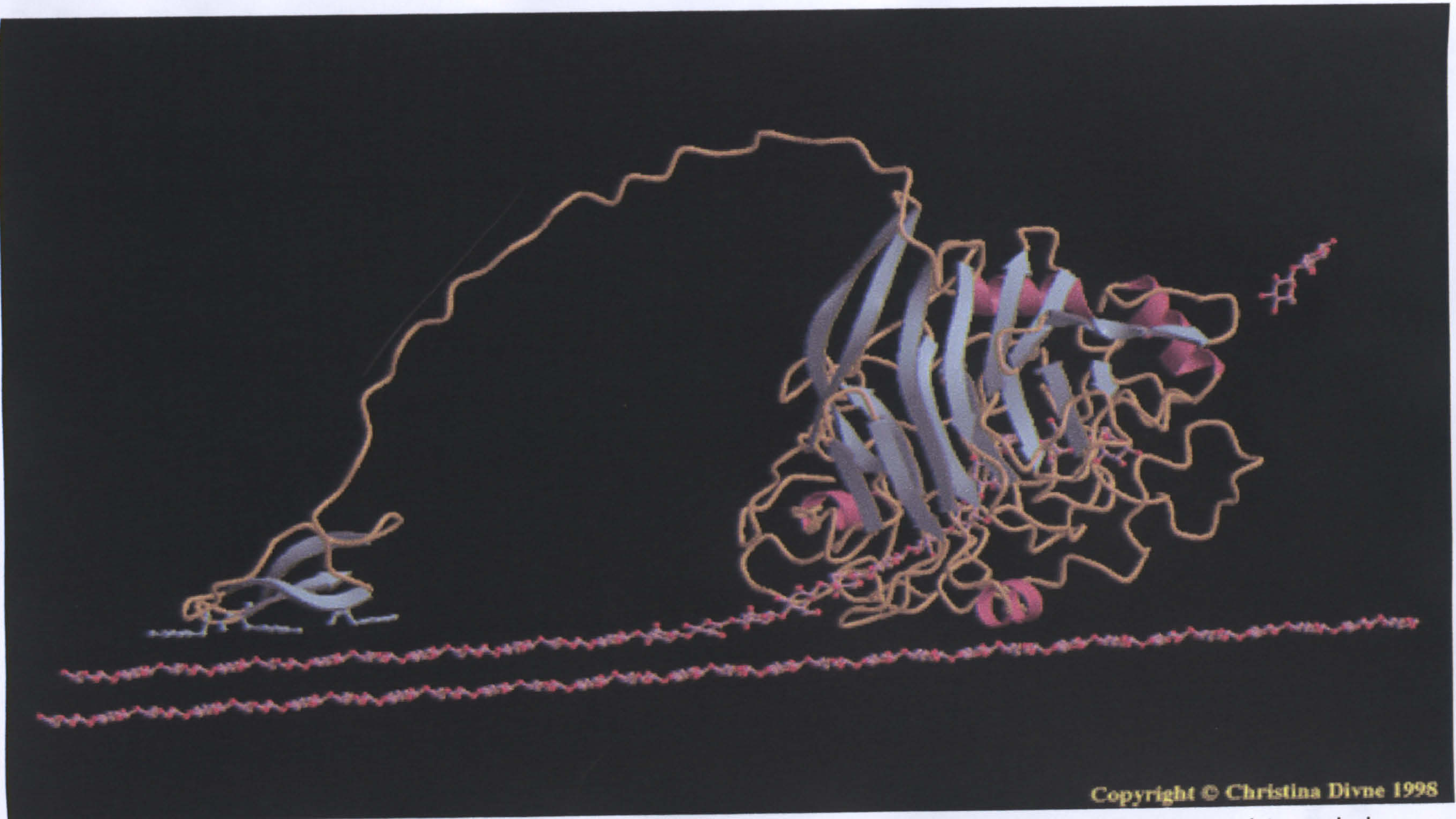


A model of intact cellobiohydrolase I (*i.e.* catalytic domain, linker and cellulose-binding domain) bound to a chain on a cellulose surface (reproduced from http://alpha2.bmc.uu.se/~cici/private/research/surf/html with the permission of Dr. Christina Divne).

1. INTRODUCTION

In recent years the use of cellulases in the textile finishing and household detergent industries has been increasing and now these industries represent the largest markets for these enzymes (Cavaco-Paulo *et al.*, 1999). Although the first use of cellulases in detergents was described by Browning in 1970, these enzymes were only introduced into textile processing during the late 1980s (Kottwitz and Upadek, 1997).

Since enzymes are highly specific and selective, they can be used to create a variety of finishing effects on cellulosic textile materials, such as enhancement of fabric surface appearance by removing fuzz fibres and pills and delivering softness benefits. These applications are known as *biofinishing* or *biopolishing* and were developed mostly in Japan during the 1980s (Chikkodi *et al.*, 1995). The most successful application of cellulases in textile processing is, however, the finishing of denims to produce the fashionable "aged" or stonewashed appearance. This washing process was discovered in the UK in the mid-1980s (Godfrey, 1996) and has gradually been replacing the traditional stonewashing process (see 2.2.2).

Enzymatic treatments of textile materials often constitute a more ecological alternative to traditional and more polluting chemical processes. Since enzyme-catalysed reactions are carried out in mild conditions, there is often a saving of chemicals and energy. Furthermore, the products obtained are natural and totally biodegradable although they may have high COD and BOD. This issue is particularly important nowadays since environmental legislation is becoming more restricting and the cost of wastewater treatment is rising. On the other hand, enzymes are often regarded as expensive reagents and since the textile industry is extremely competitive and cost conscious, there has been some resistance to the introduction of enzymatic processes.
Enzymes are true catalysts in the sense that they are not consumed during the reaction, they remain active and are therefore potentialy recyclable. Despite

this, and the concern over high cost, there have been no attempts in the textile processing industry to recycle cellulases. All the attention has so far been concentrated on developing new effects and better processes and improved cellulase compositions for achieving them.

The main objective of this research project has therefore been to study and evaluate the possibilities for recycling cellulases after their use in cotton processing. After enzymatic hydrolysis, cellulases are distributed between the treatment liquor and the substrate (cellulosic textile material). This means that cellulases can possibly be recovered from both phases. This study therefore includes:

(i) experiments to establish the activity of cellulases in used process-liquors and on the cotton substrate;

(ii) evaluation of the deactivation of cellulases by the presence of hydrolysis products such as glucose and cellobiose and by the effects of temperature and agitation;

(iii) studies of the adsorption and desorption of different cellulase components to provide information as to how cellulase mixtures must be adjusted for recycling;

(iv) attempts to separate cellulases from end-products and dyes released during processing by using ultrafiltration techniques;

(v) the use of surfactants to assist the desorption of cellulases from the textile substrate and filtration membranes.

Finally, an attempt is made to calculate the cost of recycling cellulases compared with that of using fresh enzymes for each treatment.

2. LITERATURE REVIEW

2.1 Cellulases

2.1.1 Occurrence and historical background

Cellulose, the major polysaccharide component of plant cell walls, is degraded in nature by a variety of highly specialized microorganisms, which secrete enzyme complexes (cellulases) that synergistically carry out the complete hydrolysis of cellulose (Divne *et al.*, 1994; Tormo *et al.*, 1996). Thus, cellulolytic microorganisms play an important role in the biosphere by recycling plant biomass and nutrients, maintaining soil fertility and preserving the global carbon cycle. These microorganisms can be found in all biota where cellulosic wastes accumulate (Béguin and Aubert, 1994). There is a wide variety of microorganisms that are capable of producing cellulolytic enzymes and these include fungi and bacteria, aerobes and anaerobes, mesophiles and thermophiles. Some important examples of fungi that produce cellulases are *Trichoderma* sp., *Penicillium* sp., and *Humicola insolens*. Bacteria that produce cellulases include *Cellulomonas* sp., *Thermomonospora* sp., and *Clostridium thermocellum* (Bhat and Bhat, 1997).

Interest in cellulases started almost a century ago, when Pringsheim (1912) made the first report concerning the activity of cellulases. Despite this early interest little progress on cellulase enzymology was made in the subsequent fifty years (Clarke, 1997). These enzymes did, however, receive renewed attention from Reese and co-workers, who studied the decomposition of cellulose-based US army equipment, under tropical conditions during World War II (Bhat and Bhat, 1997; Henrissat, 1994; Nevalainen *et al.*, 1991; Tomme *et al.*, 1995*a*).

With the energy crisis in the seventies, much attention was devoted to this class of enzymes because of their potential use for biomass degradation into soluble

and fermentable sugars, which provide a source of renewable energy and chemicals. At the same time developments in biotechnology were making enzymes available on a large scale.

Cellulose degrading enzymes have an ecological and commercial importance since they participate in the recycling of plant biomass and are established in a number of industries, which will be described in a further section (2.2.1). Apart from their wide range of industrial applications, cellulases are very interesting from the biochemical and structural point of view. Consequently these enzymes continue to attract the interest of biotechnologists, X-ray crystallographers, biochemists and molecular biologists as well as that of technologists looking for improved industrial processes.
During all these years of research much progress has been made and an extensive literature concerning the biodegradation of cellulosic materials is now available.

2.1.2 Classification and nomenclature of cellulase structures and families

Enzyme Commission classification

The efficient biodegradation of cellulose requires the interaction of several distinct enzymes, which catalyse the hydrolytic cleavage of 1,4-β-glycosidic bonds. They are known as 1,4-β-glycoside hydrolases or more commonly simply as cellulases. Enzymes, in general, are grouped and classified according to a scheme which was established by the first Enzyme Commission (EC) set up by the International Union of Biochemistry (IUB) in 1961 (Nomenclature Committee IUB, 1984). The EC numbers contain four elements separated by points. The first element is concerned with the six main divisions or classes of enzymes: 1-oxidoreductases, 2-transferases, 3-hydrolases, 4-lyases, 5-isomerases and 6-ligases. The second element indicates the subclass and is

related to the type of bond at which the enzyme acts. The third element gives the sub-subclass and the fourth element is the serial number of the enzyme in its sub-subclass. Cellulases belong to the class of hydrolases (3), which act on glycosidic bonds (subclass 2), and to the sub-subclass (1) of glycosylases of *O*-glycosil compounds. Thus, endoglucanases (EGs) or endo-1,4-β-D-glucan-4-glucanohydrolases are EC 3.2.1.4 and exoglucanases, cellobiohydrolases (CBHs) or 1,4-β-D-glucan cellobiohydrolases are EC 3.2.1.91. Cellobiase or β-glucosidase (β-D-glucoside glucohydrolase) is EC 3.2.1.21 (Baker *et al.*, 1998; Beldman *et al.*, 1988; Bok *et al.*, 1998; Henrissat *et al.*, 1985; Hoshino *et al.*, 1999; Kleywegt *et al.*, 1997; Klyosov, 1990; Lee and Brown Junior, 1997; Medve *et al.*, 1998*a*).

Classification of catalytic domains

Most cellulases, like many other enzymes involved in the hydrolysis of insoluble polysaccharides, are modular enzymes with a distinct cellulose binding domain (CBD), joined to the catalytic domain (core) by an extended linker peptide that is often glycosylated (see Figure 2.1) (Gilkes *et al.*, 1992; Henriksson *et al.*, 1997, Kotiranta *et al.*, 1999; Linder and Teeri, 1997; Ong *et al.*, 1993; Srisodsuk *et al.*, 1998; Tomme *et al.*, 1995*b*).

The catalytic domain is larger than the CBD and represents more than 70% of the total protein (Bhat and Bhat, 1997). Most linkers are rich in proline and hydroxyaminoacids (often alanine and glycine residues). This composition, and the regular arrangement of the residues, probably imparts structural stability through extensive hydrogen bonding while allowing the necessary flexibility. Linker structure and function demands an exposed position in the molecule and glycosylation also contributes to stability in an aqueous environment and protects against proteolysis. (Tomme *et al.*, 1995*a*). It is reasonable to assume that linkers allow flexible connection between the two domains and ensure the optimal distance for domain interaction (Kotiranta *et al.*, 1999).

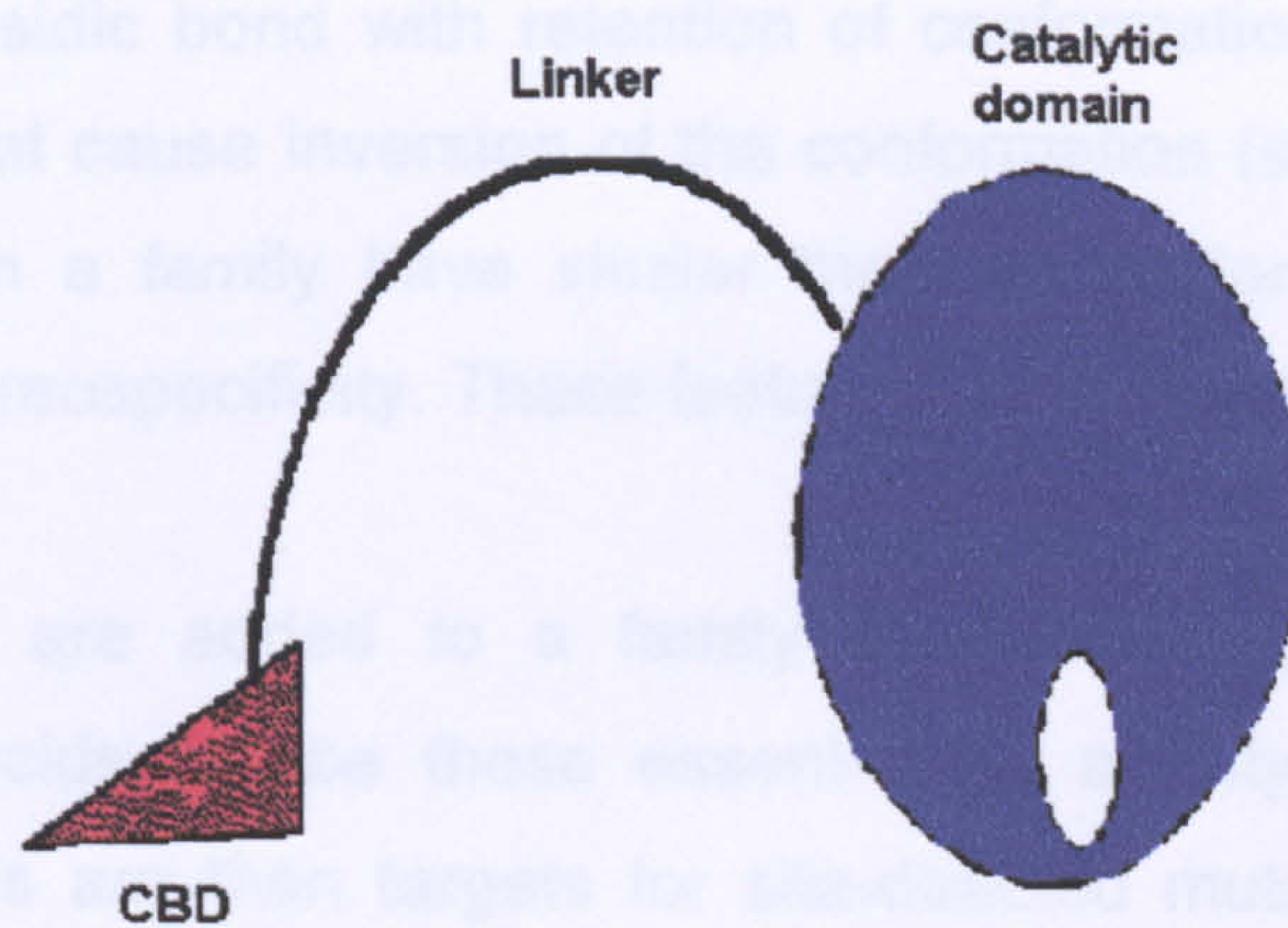


Figure 2.1 Schematic representation of the two-domain structure typically found in cellulases.

The EC classification does not reflect the structural features of enzymes and consequently a new classification of families (identified by Arabic numbers) based on amino acid sequence similarities, was proposed by Henrissat (1991) to complement the IUB (EC) nomenclature of glycosyl hydrolases (Nomenclature Committee IUB, 1984). This new classification is intended to reflect the structural features of these enzymes, to help reveal the evolutionary relationships between them and to provide a convenient source of mechanistic information. A rapidly growing number of glycosyl hydrolase genes are being sequenced and the number of three-dimensional structures being solved is increasing. Consequently a classification that better reflects sequence and hence structure is certainly more useful. This type of classification has the advantage that a protein or a gene translation, or even a domain, can be classified before knowing its enzymatic activity (Henrissat and Bairoch, 1993). As more sequences become available, the sequence-based classification of glycosyl hydrolases was updated (Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996).

All glycoside hydrolases can be divided into two large groups: those that hydrolyse the glycosidic bond with retention of conformation at the anomeric centre and those that cause inversion of the conformation (see Figure 2.2). All catalytic domains in a family have similar three-dimensional structures and exhibit the same stereospecificity. These features are illustrated in Table 2.1.

As more enzymes are added to a family of catalytic domains, the fully conserved amino acids will be those essential for activity. Fully conserved carboxy amino acids are then targets for site-directed mutations. In general, mutation of such a residue should lower the activity of an enzyme. Molecular genetics, chemistry, biochemistry and X-ray crystallography are revealing in great detail the catalytic mechanism of a variety of 1,4-β glycanases, thereby allowing an understanding of determinants of substrate specificity and mode of action. The availability of high-resolution structures has yielded clues as to how the specificity for each substrate is controlled and has facilitated the engineering of cellulases with increased activity towards native celluloses (Spezio *et al.*, 1993).

One method of identifying the amino acids, which are important for catalysis, involves kinetic analysis of single residue mutant forms of the enzyme. In this way, a direct and quantifiable assessment of the contribution of each individual amino acid toward catalysis can be made. These methods have enabled many studies that have furthered the understanding of cellulase hydrolysis mechanisms (Damude *et al.*, 1995; Davies *et al.*, 1997; Mattinen *et al.*, 1997*a*; Mattinen *et al.*, 1997*b*).

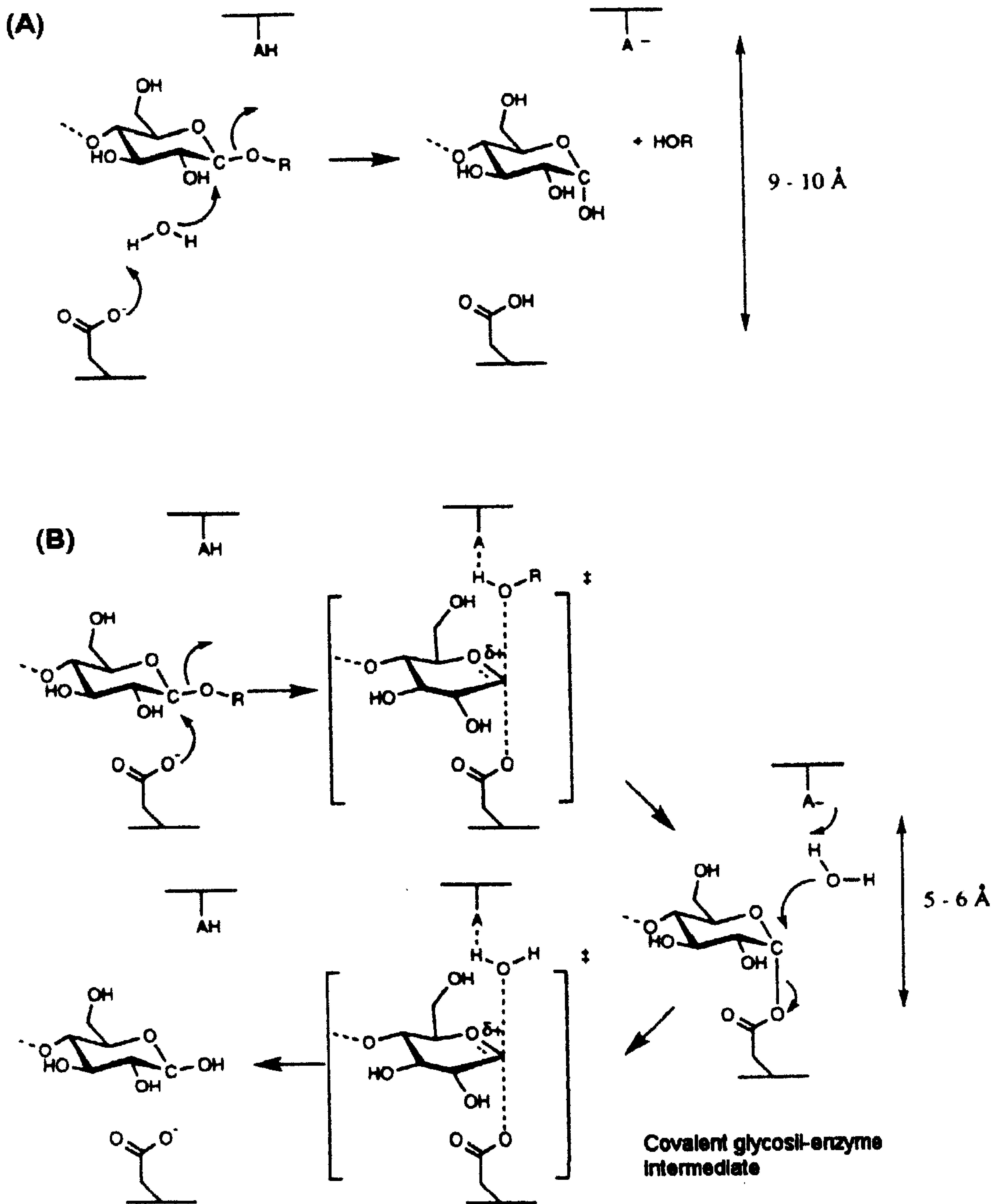


Figure 2.2 Reaction mechanisms for the hydrolysis of β-glycosides. (A) The single-displacement mechanism with inversion of configuration at anomeric carbon atom of the hydrolysed glycoside. (B) The double-displacement reaction mechanism for retaining enzymes (reproduced from Davies, 1998).

CBDs have also been grouped into several families based on similarities in their amino acid sequences. Each family has been named using Roman numerals to avoid confusion with Arabic numbers used for catalytic domain classification (Tomme *et al.*, 1995*b*). This classification is now in general use. In Table 2.1 the classification and families of CBDs and catalytic domains of the most widely used cellulases are listed. More detailed information about these enzymes may be found in the literature (Béguin and Aubert, 1994; Henrissat, 1994; Linder and Teeri, 1997; Ohmiya *et al.*, 1997; Reinikainen, 1994; Tomme *et al.*, 1995*a*; Tomme *et al.*, 1995*b*).

CBDs in family I are the smallest (33-40 amino acid residues with similar highly conserved sequences) and occur only in fungal cellulases. Family II CBDs are much larger in size. Although there is an overall functional similarity between all CBDs, their specific properties are not always identical, even among members of the same family.
Some CBD structures are already solved. The first to be published was that of the family I CBD found in CBHI of *Trichoderma reesei* (Kraulis *et al.*, 1989). The second was that of the family II, CBD of a mixed function xylanase-glucanase (Cex) secreted by the bacterium *Cellulomonas fimi* (Xu *et al.*, 1995).

It may be seen from Figure 2.3 that CBDs of family I and II have different sizes and topologies but their binding faces are very similar. Both proteins are composed of antiparallel β-sheets that form a flat binding surface containing three exposed aromatic residues, which seem to mediate the binding interaction with cellulose (Johnson *et al.*, 1996*a*). The differences in size and topology may influence the affinity, specificity or desorption behaviour of the various CBDs on different substrates.

Table 2.1 Summary of the classification of CBDs and catalytic domains of some cellulases (Davies *et al.*, 1995; Henrissat and Bairoch, 1996; Ohmiya *et al.*, 1997; Teeri, 1997; Tomme *et al.*, 1995*b*).

Enzyme name	Organism	CBD		Catalytic domain	
		Family	Size[a]	Family	Stereospecificity
CBHI	*H. insolens*	I	36	7	Retaining
CBHII	*H. insolens*	I	36	6	Inverting
EGI	*H. insolens*	I	36	7	Retaining
EGII	*H. insolens*	I	36	5	Retaining
EGV	*H. insolens*	I	33	45	Inverting
CBHI	*T. reesei*	I	36	7	Retaining
CBHII	*T. reesei*	I	36	6	Inverting
EGI	*T. reesei*	I	33	7	Retaining
EGII	*T. reesei*	I	36	5	Retaining
CbhA	*C. fimi*	II	104	6	Inverting
CbhB	*C. fimi*	II	106	48	Inverting
CenA	*C. fimi*	II	106	6	Inverting
E1	*T. fusca*	II	96	9	Inverting
E2	*T. fusca*	II	96	6	Inverting
E5	*T. fusca*	II	104	5	Retaining

[a] The sizes of CBDs are given in numbers of amino acids residues.

The influence of the CBDs on cellulase activity is not well understood and most information about the role of CBDs has been obtained by removal, domain exchange or site-directed mutagenesis of the CBDs (Linder and Teeri, 1997). It is known, however, that CBDs potentiate the hydrolysis of insoluble cellulose, since removal of the CBD reduces the activity on insoluble substrates, but has little effect on the hydrolysis of soluble substrates (Lee and Brown Junior,

1997). It has been postulated that this is because the CBD increases the concentration of the catalytic domain near the substrate, thereby increasing hydrolytic activity (Linder and Teeri, 1997).

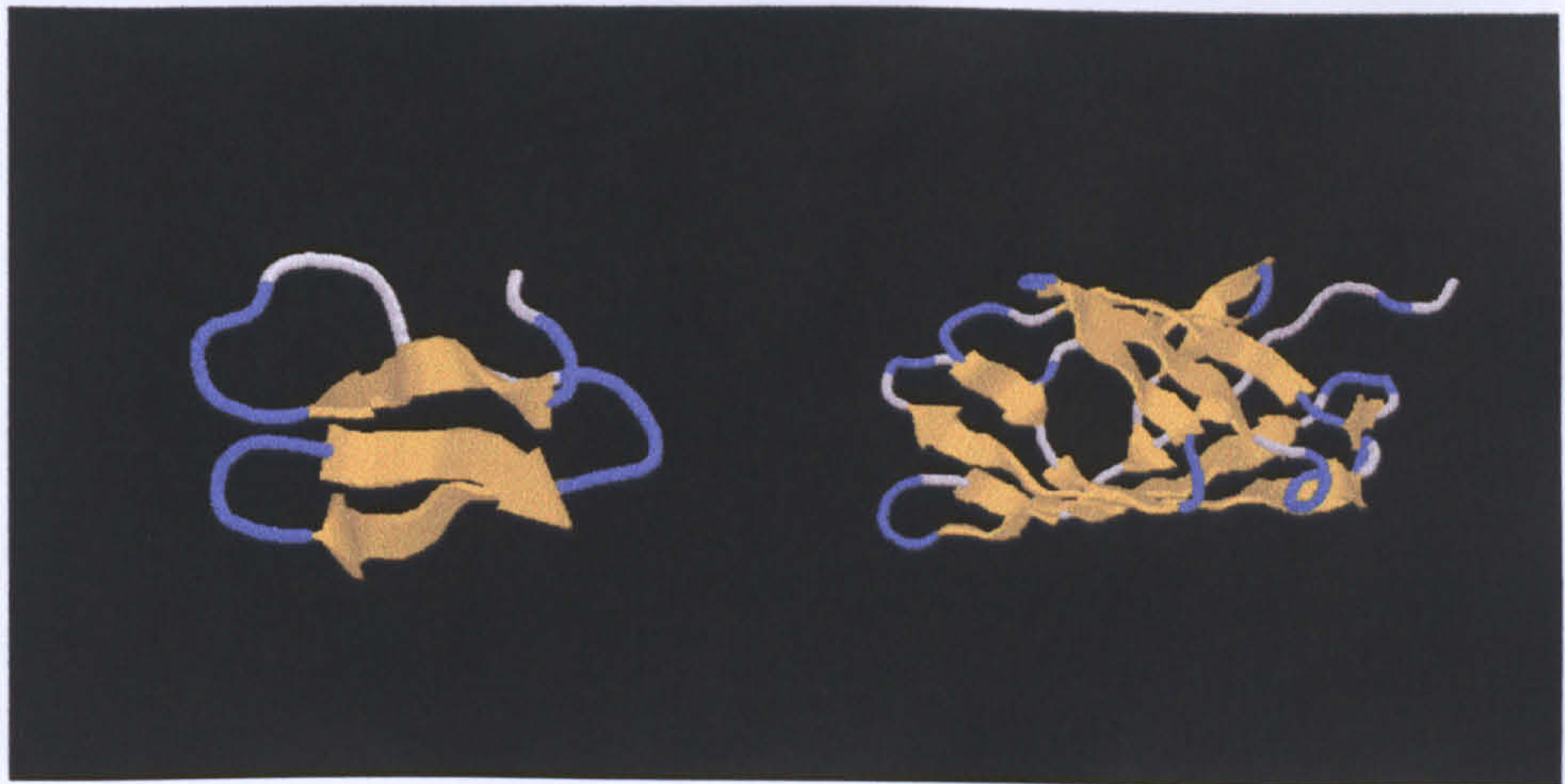


Figure 2.3 Cartoon three-dimensional structures of the CBDs of Cellobiohydrolase I from *Trichoderma reesei* (left) and Cex from *Cellulolomonas fimi* (right). Figure drawn using the program Rasmol with structures obtained from Brookhaven Protein Data Bank (http://www.rcsb.org/pdb).

It has been shown that some CBDs of family II can disrupt the surface of cellulosic fibres and release fine particles from cotton and microcrystalline cellulose (Boraston *et al.*, 1998). Similar results, however, have not so far been observed with other CBDs (Linder and Teeri, 1997).

Many studies have been made using the site directed mutagenesis and chemical modification approach to investigate the importance of aromatic residues on ligand binding (Damude *et al.*, 1995; Davies *et al.*, 1995; Koivula *et al.*, 1998; Zhang and Wilson, 1997). The aromatic tyrosine or tryptophan rings are thought to provide a hydrophobic driving force for binding by stacking directly against the pyranose rings of crystalline cellulose (Johnson *et al.*, 1996*b*). Mutational analysis of CBDs in family II also supports the conclusion that exposed tryptophan chains are involved in cellulose recognition.

New nomenclature

In recent years, the identification of new enzymes, as well the elucidation of their structure and mechanism, has led to the necessity for re-grouping the enzymes and giving them new designations in order to achieve better enzyme identification and to simplify comparison between the systems of enzymes produced by different microorganisms. New nomenclature has therefore been proposed for enzymes that hydrolyse the polysaccharides in the cell-walls of plants (Henrissat *et al.*, 1998). The scheme is based on the classification of the catalytic domains of glycoside hydrolases into families of related amino acid sequences. The new designation for an enzyme indicates its family, its three-dimensional fold-structure and its stereospecificity of hydrolysis because a family has these characteristics in common. As examples, CBHI is now called Cel7A, CBHII is Cel6A and EGI is Cel7B.

In a recent publication, Boraston *et al.* (1999) proposed the term "carbohydrate binding module" (CBM) instead of cellulose binding domain (CBD), because these domains or modules are also present in other polysaccharidases and have affinity for carbohydrate ligands other than cellulose. New designations for the classification and organization of the CBDs are also proposed in this reference. It is already possible to find the term CBM for designating the binding domain of some enzymes in recent literature (Charnock *et al.*, 2000; Hachem *et al.*, 2000; Parsiegla *et al.*, 2000; Ponyi *et al.*, 2000) although use of the term CBD still continues (Brun *et al.*, 2000).

Although these new systems of cellulase nomenclature are already in use (Davies *et al.*, 1998; Koivula *et al.*, 1998; Mackenzie *et al.*, 1998; Parsiegla *et al.*, 2000; Zou *et al.*, 1999), the "old system" is used throughout this thesis to avoid possible confusion.

Continual updating of protein sequences, structures, functions and mechanisms can be found in the websites http://www.expasy.ch/sprot (SwissProt+TrEML); http://www.rcsb.org/pdb (Brookhaven Protein Data Bank) and http://www.expasy.ch/enzyme, respectively.
The server CAZy (http://afmb.cnrs-mrs.fr/~pedro/CAZY) also describes the families of structurally related catalytic domains of enzymes that degrade glycosidic bonds.

2.1.3 The cellulolytic systems produced by the fungi *Trichoderma reesei* and *Humicola insolens*

The cellulase system of the filamentous fungus *Trichoderma reesei* has been found to be one of the most effective for the hydrolysis of cellulosic materials (Ellouz *et al.*, 1987; Medve *et al.*, 1994; Rouvinen *et al.*, 1990). For this reason, it has been applied in some important industrial applications as well in numerous studies. These studies have provided much new information and the cellulase system secreted by this fungus is now the best understood in terms of enzymology, substrate specificity, gene sequence and synergism (Banka *et al.*, 1998; Biely *et al.*, 1991; Lee *et al.*, 1997).

The cellulolytic complex of *T. reesei* is composed of two cellobiohydrolases (CBHI and CBHII) at least four endoglucanases (EGI, EGII, EGIII and EGV) and one β-glucosidase (Heikinheimo *et al.*, 1998; Medve *et al.*, 1998*a*; Srisodsuk *et al.*, 1998). Some properties of each cellulase component are summarized in Table 2.2.

Table 2.2 Some properties and schematic structural organization of the cellulase system produced by *T. reesei* (Reinikainen, 1994; Srisodsuk, 1994).

Enzyme	% in the mixture	AA[b]	Isolectric point (pI)	Molecular mass (kDa)	Structural organization[d] (N → C)
CBHI	60	497	3.5-4.2	59-68	430 / 31 / 36
CBHII	20	447	5.1-6.3	50-58	36 / 44 / 365
EGI	10	437	4.6	50-55	368 / 33 / 36
EGII	1	397	5.5	48	36 / 34 / 327
EGIII	nd[a]	218	7.4	25	218
EGV	nd[a]	225	2.8-3.0	23[c]	166 / 23 / 36
β-glucosidase	nd[a]	713	8.7	75	-

[a] not determined;

[b] Number of amino acid residues;

[c] The molecular mass was calculated from amino acid sequence;

[d] □ - catalytic domain; ▒ - linker; ■ - CBD. Letters C and N denote C and N terminal, respectively.

Humicola insolens produces at least seven cellulases: two cellobiohydrolases (CBHI and CBHII) and five endoglucanases (EGI, EGII, EGIII, EGV and EGVI) (Varrot *et al.*, 1999*a*). Three-dimensional X-ray structures are known for CBHII, EGI and EGV from *H. insolens*. With the exception of EGV, all the enzymes from *H. insolens* display high levels of sequence similarity with their homologues from *T. reesei*.

Table 2.3 Some properties of the cellulase system produced by *H. insolens* (Schülein *et al.*, 1993).

Enzyme	Isolectric point (pI)	Molecular mass (kDa)	CBD
CBHI	4.5	72	Yes
CBHII	4.6-5.2	65	Yes
EGI	5.5	50	No
EGII	7.0	50	Yes
EGIII	5.2	26	No
EGV	5.2	43	Yes
EGVI	5.0	43	No

2.1.4 Cellulase adsorption

Since native cellulosic materials are water-insoluble, solid substrates, the cellulose-cellulase system is heterogeneous and the adsorption of enzyme molecules on susceptible sites of the cellulose surface is a prerequisite step for subsequent catalytic reaction. Knowledge about cellulase adsorption is of great importance since it aids understanding of the mechanism of hydrolysis, which may then lead to informed optimisation of industrial processes. Information about the adsorption of individual cellulolytic components is necessary to evaluate possible correlations between binding of each component and the synergism in their catalytic action during substrate hydrolysis. Furthermore, study of the distribution of different cellulase components between supernatant and residual substrate could lead to more effective recycling of cellulases (see 2.3.1).

The number of variables involved makes cellulase adsorption a rather complex subject. The heterogeneity of cellulose (crystalline and amorphous) and its possible change during hydrolysis, together with the multi-component cellulolytic mixture, are some examples of the system complexity. Others factors affecting adsorption are pH, temperature, ionic strength, presence of surfactants, degree of agitation and mass-transfer effects (Kyriacou *et al.*, 1988; Medve *et al.*, 1998*a*; Nidetzky *et al.*, 1994*a*; Ryu *et al.*, 1984; Steiner *et al.*, 1988). The studies reported in the literature are often difficult to conciliate, since the reaction conditions vary greatly from study to study. Thus careful examination of data interpretation is necessary.

The dominating interactions during protein adsorption to uncharged carbohydrate polymers, such as cellulose, are mainly hydrogen bonding (Kyriacou *et al.*, 1988), hydrophobic interactions, or van der Waals forces (Medve *et al.*, 1997). Recent studies of the structure and composition of the CBDs of cellulases demonstrated the presence of conserved, exposed aromatic amino acid residues on one side of the CBDs (tyrosines on family I CBD and tryptophan on family II) which may be involved in the interaction of the cellulases with the cellulose surface (Carrard and Linder, 1999; Henriksson *et al.*, 1997; Medve *et al.*, 1997; Tomme *et al.*, 1995*b*).

Influence of temperature

The influence of temperature on cellulase adsorption is well documented but the results are conflicting. Most of the studies have been carried out at low temperature (2-10 °C) in order to minimize hydrolysis. In realistic conditions, however, the hydrolysis is performed at 50 °C (usually the optimum temperature for cellulase activity). During hydrolysis more cellulose surface becomes exposed to cellulase adsorption.

Steiner *et al.* (1988) investigated the adsorption of *T. reesei* cellulases on Avicel (a commercially available microcrystalline cellulose) and bleached spruce

sulphite pulp at 2 °C and 50 °C. It was found that at 2 °C the adsorption process is very fast (half of the maximally adsorbed enzyme was bound within the first 1-2 minutes) and adsorption equilibrium was reached after about 30 minutes. At 50 °C, however real equilibrium could never be established, despite the adsorption rate at this temperature being faster than at 2 °C.

An adsorption study with fractionated *T. reesei* cellulase components on purified cellulose (Kyriacou *et al.*, 1988) showed that increasing the temperature increased the level of saturation for EG I, EG II and EG III, but for CBH I it had a negligible effect. Using elevated temperatures can enhance the initial adsorption by opening space between microfibrillar chains, thereby increasing the number of available adsorption sites. In later work (Kyriacou *et al.*, 1989), it was found that as the temperature increased from 5 to 50 °C, the equilibrium adsorption of all cellulase components decreased.

In Ryu's study (Ryu *et al.*, 1984) of adsorption of cellulase components on Avicel at different temperatures it was also demonstrated that the equilibrium adsorption decreases with increasing temperatures.

Ooshima *et al.* (1983) showed, for cellulases ex *T. viride*, that the lower the temperature, the more preferential the adsorption for endoglucanases compared with cellobiohydrolases. At higher temperatures the situation was reversed.

Adsorption studies by Moloney and Coughlan (1983) using *Talaromyces emersonii* cellulases on Solka Floc (see Table 2.4, legend) at 4, 45 and 60 °C showed that despite the rapid, initial adsorption (first minute) being greater at the higher temperatures (45 and 60 °C), this was followed by a gradual release of enzyme to solution. By contrast, at 4 °C the binding of the enzyme continued to increase with time.

Van Wyk (1997*a*) found that the relative rate of adsorption of *Penicillium funiculosum* cellulase increased with increasing temperature.

Adsorption models

Various models have been used to describe the cellulase adsorption process. The Langmuir-type isotherm has been the most widely applied (Beldman *et al.*, 1987; Bothwell *et al.*, 1997*a*; Gerber *et al.*, 1997; Kim *et al.*, 1997; Kim *et al.*, 1998; Kyriacou *et al.*, 1988; Ooshima *et al.*, 1983; Ooshima *et al.*, 1990).
Some authors have applied a combination of Langmuir, Freundlich and other models to the adsorption of CBH I and CBH II from *T. reesei* on microcrystalline cellulose (Medve *et al.*, 1997). They found that the experimental data were best described by the Langmuir model with two types of adsorption sites or by a combined Langmuir-Freundlich model. The same models were used in later work with *T. reesei* CBH I and EG II (Medve *et al.*, 1998*a*).

Reversibility of adsorption

Another controversy found in cellulase adsorption studies concerns the irreversible or reversible nature of the binding of the enzymes to the substrate. This question is of key importance since many potential applications depend on enzyme recycling.
The reversibility of adsorption of *Trichoderma viride* cellulases was examined by dilution of the supernatant under conditions of about 50% saturation (Beldman *et al.*, 1987). It appears that at least a part of the enzyme molecules were irreversibly adsorbed on cellulose chains even after prolonged desorption time. Kyriacou and co- workers (1989) studied the reversibility of adsorption of *T. reesei* cellulases and observed that upon dilution no release of bound enzymes was detected.
The adsorption of a bacterial cellulase (CenA from *Cellulomonas fimi*) on crystalline cellulose is very fast and no detectable protein desorption was observed during the following 16.7 h (Gilkes *et al.*, 1992).

Since it is known that CBDs play an important role in cellulase adsorption, many studies have been made with isolated CBDs and truncated enzymes as well as with entire enzymes.

Jervis *et al.* (1997) concluded that the CBDs of bacterial cellulases from *C. fimi* (CenA and Cex CBDs, family II) bind irreversibly to crystalline cellulose because no desorption of these CBDs was observed after dilution. Boraston *et al.* (1998) found similar results with Cex CBD and Bothwell *et al.* (1997*b*) reported irreversible adsorption for the endoglucanase E3 and E5 from *Thermomonospora fusca* (CBDs of family II). These workers also found completely reversible binding for CBH I of *T. reesei* (family I CBD). The latter is consistent with reversible binding of the isolated CBD of CBH I *T. reesei* demonstrated by Linder and Teeri (1996). More recent investigations of the reversibility of binding of the isolated CBD of *T. reesei* CBH II (Carrard and Linder, 1999) showed that, despite the apparent similarities in the binding affinities of the CBDs of CBH I and CBH II, the CBD of CBH II could not be desorbed from the substrate by buffer dilution. Desorption could, however be induced by raising the temperature. In contrast, it was observed that the CBH II CBD bound reversibly to chitin. Variants of this CBD, with specific mutations that increased its similarity to CBH I CBD, showed reversible binding to cellulose.

Thus, the findings described above suggest that the reversibility or irreversibility of cellulase adsorption is closely related to the fungal or bacterial origin of the cellulases and in particular, those with family I CBDs tend to adsorb reversibly whereas those with family II CBDs adsorb irreversibly. Substrate properties and reaction conditions can, however, also influence the reversibility of this process.

2.1.5 Hydrolysis of cellulose by cellulolytic enzymes

All cellulolytic enzymes share the same chemical specificity for 1,4-β-glycosidic bonds, which they cleave by a general acid-catalysed hydrolysis, leading to

either inversion or retention of conformation at the anomeric carbon (see section 2.1.2, Figure 2.2). The different cellulase classes, however, hydrolyse cellulose in specific ways.

These different modes of action are commonly described as endo- and exo-types of attack (Figure 2.4). A typical endoglucanase cleaves bonds along the length of the cellulose chains, resulting in rapid decrease in the degree of polymerisation (DP) of the substrate (Fontaine *et al.*, 1997; Ramos *et al.*, 1993*a*). Because glucan chains can remain associated with the rest of the crystal after a single bond cleavage at the surface, it takes a relatively long time before soluble products are observed after an endo-type attack. Exoglucanases (cellobiohydrolases) are currently thought to be processive enzymes, initiating their action from the ends of the cellulose chains (Baker *et al.*, 1998; Teeri *et al.*, 1998). An increasing amount of evidence indicates that different CBHs have opposite chain-end specificities. CBHI attacks reducing ends whereas CBHII attacks nonreducing ends (Teeri, 1997). It was observed that CBHI from *T. reesei* as well *T. fusca* exoglucanases E4 and E6 prefer the reducing end of oligosaccharides, while CBHII and E3 act at the nonreducing end (Barr *et al.*, 1996). In this work, the authors propose that all retaining exocellulases will cleave from the reducing end. Hydrolysis by retention is a two-step process, passing through an intermediate where part of the substrate (the nonreducing end portion) is covalently bound to the enzyme. If a retaining exocellulase were to attack the nonreducing end, it would form a covalent bond to cellobiose and release the residual cellulose chain. Cleavage from the reducing end seems to be more likely, since the cellulosic chain is bound in the active site.

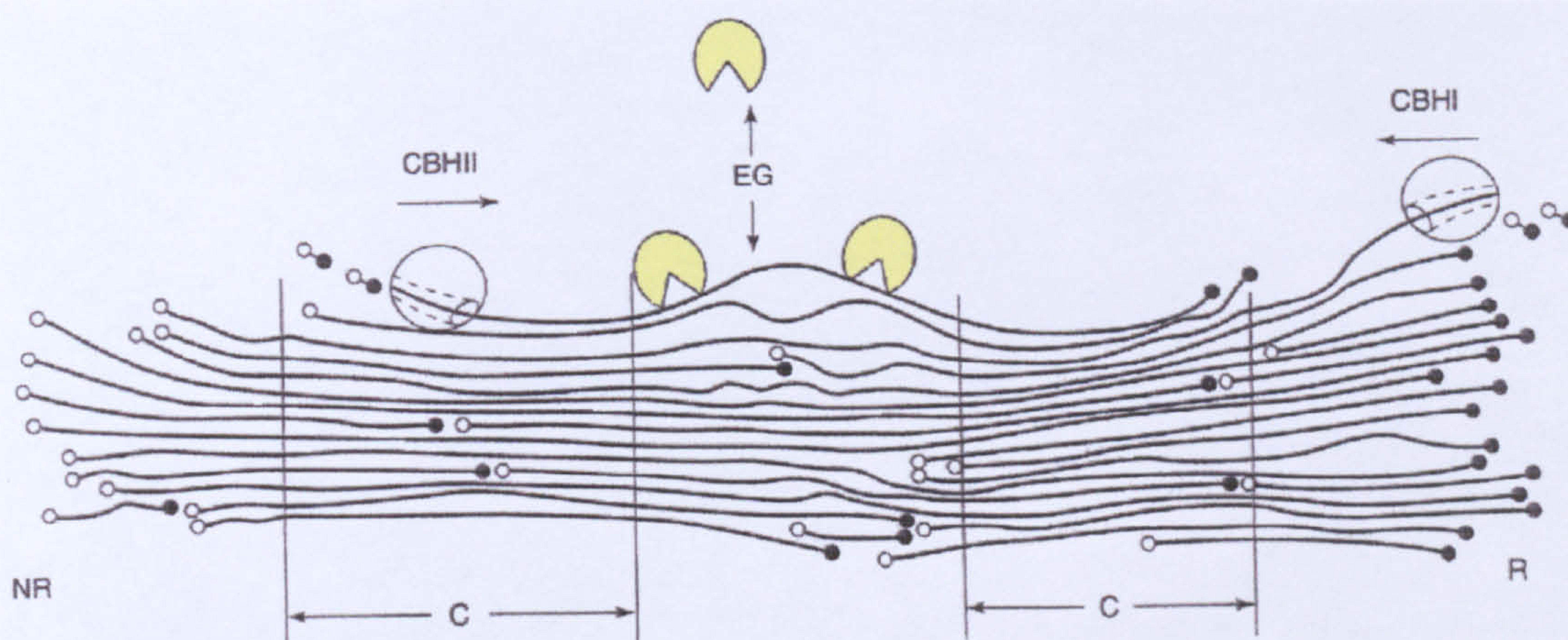


Figure 2.4 Schematic representation of the hydrolysis of crystalline cellulose by different EGs and CBHs. The filled circles, denoted R, represent reducing ends and the open circles, denoted NR, represent the non-reducing ends. C defines the highly ordered crystalline region (reproduced from Teeri, 1997).

There is also evidence that in the absence of EGs, CBHII displays endo activity (Boisset *et al.*, 2000; Ramos *et al.*, 1999*a*). CBHs can attack the crystalline parts of the substrate, producing primarily cellobiose and decreasing the substrate DP only very slowly.

The main structural difference between endo and exocellulases determines the degree to which their active sites are accessible to substrate (Spezio *et al.*, 1993). The three-dimensional structure of a cellobiohydrolase reveals that the active site is tunnel-shaped whereas those of endoglucanases are open clefts, allowing easier access to internal glycosidic bonds (Figures 2.4 and 2.5). This may explain the differences in the modes of action of the two classes of enzymes.

Finally, β-gucosidase completes the hydrolytic process by catalysing the hydrolysis of cellobiose to glucose or by removing glucosyl residues from the nonreducing end of soluble oligosaccharides (Ramos *et al.*, 1993*a*).

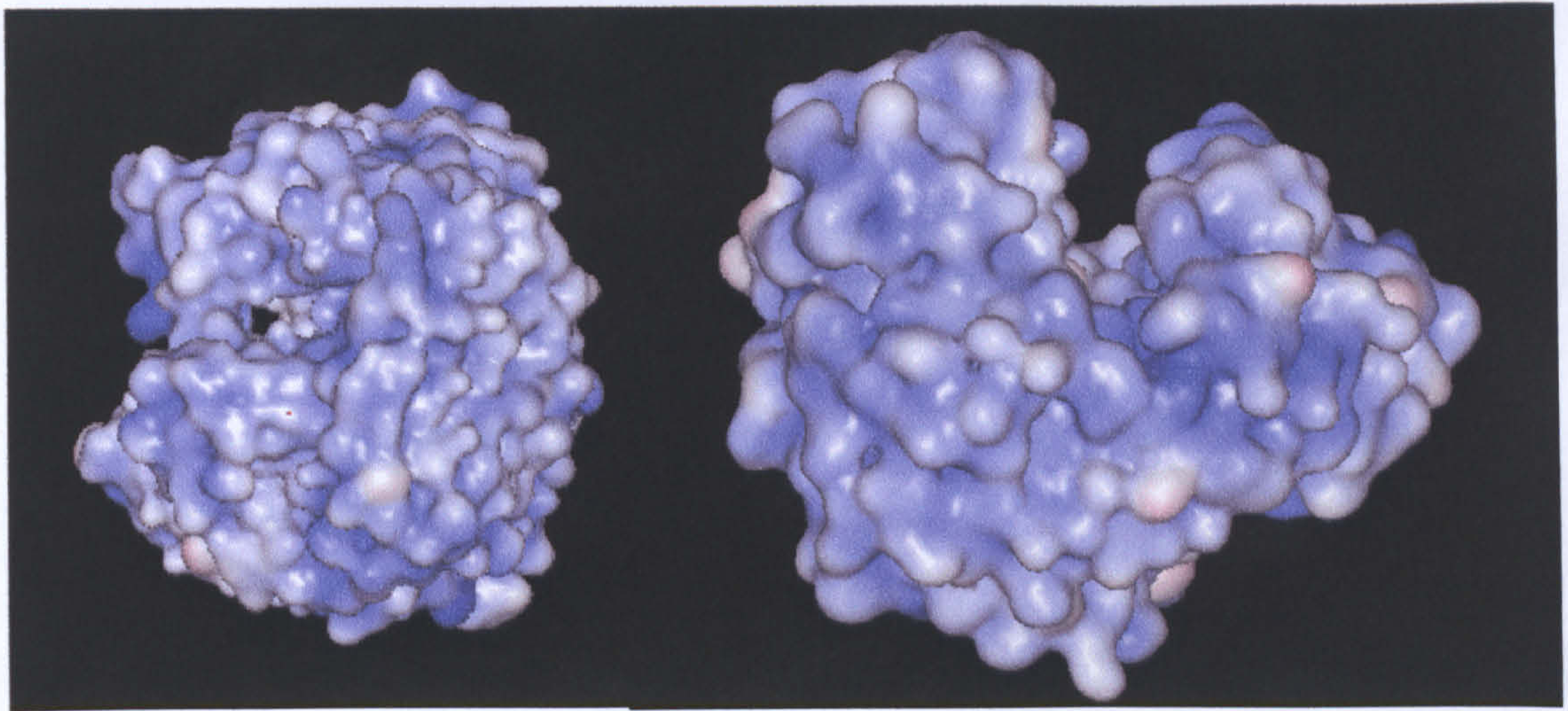


Figure 2.5 Surface images of an exoglucanase (*Trichoderma reesei* CBHII-left) and endoglucanase (*Thermomonospora fusca* E2-right) to illustrate the different site topologies. Figure drawn using program WebLab Viewer with structures obtained from Brookhaven Protein Data Bank (http://www.rcsb.org/pdb).

Stålberg and co-workers (1991) proposed a model for the action for the two cellulase domains. The core domain contains the active site buried in a cleft with subsites for several glucose units in a single cellulose chain. In order to bind a chain on the cellulose surface, the enzyme needs to break a number of interchain, non-covalent bonds, the number of which is dependent on the degree of crystallinity. This will give a bias for the core towards amorphous and or chain end regions. The binding domain binds preferably to the crystalline regions because it interacts with more than one chain without the need to break any interchain bonds. The overall binding specificity of the complete enzyme is determined by the relative affinities of the domains and the number of available binding sites.

Methods for characterizing and monitoring cellulolytic hydrolysis on different substrates

A wide range of different model substrates and many different assays have been developed and used to characterize individual enzymes and total enzyme systems. Some examples of the methods for measuring cellulase activity are summarized in Table 2.4.

Numerous investigations have been made over many years concerning enzymatic hydrolysis of cellulose. These studies have been mainly for improving understanding of how the different cellulase components act in the presence of each other, or in isolation, on various types of cellulosic substrates. Since hydrolysis and adsorption are closely related processes, the hydrolysis is also influenced by the same variables enunciated for the adsorption of cellulases (section 2.1.4).

Decline in the rate of hydrolysis with time

It has been shown that, over a range of enzyme and substrate concentrations, a logarithmic decline in the hydrolysis rate occurs, even where more accessible substrates are used (Mooney *et al.*, 1998; Ramos *et al.*, 1993*a*; Zhang *et al.*, 1999). This has been attributed to various factors such as deactivation of cellulase by shearing forces, thermal effects, irreversible adsorption, and end-product inhibition of the cellulase complex.

Table 2.4 Substrates and assays commonly used to measure cellulase activities (Bhat and Bhat, 1997; Enari and Markkanen, 1977; Reinikainen, 1994; Teeri, 1997).

Enzyme	Substrate	Assay
Total cellulase (TC)	• Cotton	• Solubilization: weight and tensile strength loss; reducing sugar released; estimation of cellulose residue; cotton reducing power
	• FP, Avicel[a] and Solka Floc[b], BMCC[c]	• Release of reducing sugars; determination of DP
	• Amorphous cellulose	• Decrease in turbidity
	• Dyed celluloses	• Release of dyed soluble fragments
Cellobiohydrolases (CBHs)	• Cellulosic substrates (see above)	• See above
	• Substituted (with chromophoric, fluorogenic or radioactive labels) and unsubstituted cellooligosaccharides	• Analysis of sugars by HPLC; release of chromophore; increase of reducing power
Endoglucanases (EGs)	• Cellulosic substrates (see above)	• See above
	• CMC[d]	• Release of reducing sugars
	• HEC[e]	• Decrease in viscosity
β-glucosydase or cellobiase	• Cellobiose	• Release of glucose
	• *o*- or *p*-nitrophenyl-β-D-glucoside	• Release of *o*- or *p*-nitrophenol
	•Cellooligosaccharides	• Increase in reducing power

Table 2.4 (Legend)

[a] Avicel is a commercial microcrystalline cellulose produced from highly purified spruce and hemlock pulp. The pulp is diced into small pieces, a mineral acid bath frees the crystallites from cellulose matrix, and the mixture is cooked for 45 minutes, solidified into a cake by filtration, and then spray dried (Fan *et al.*, 1980);

[b] Solka Floc is a commercial name for hammer-milled sulphite pulp. It is composed of crystalline and amorphous celluloses. It is relatively pure, containing at least 99.5% cellulose, and it contains only small amounts of extraneous material such as lignin, hemicellulose and ash (Fan *et al.*, 1980);

[c] Bacterial microcrystalline cellulose.

[d] Carboxymethylcellulose;

[e] Hydroxyethylcellulose.

Ooshima *et al.* (1991) have suggested that this is due to a loss of synergistic action between endoglucanases and cellobiohydrolases. These authors proposed an equation, which describes the decline in hydrolysis rate in terms of adsorbed enzyme concentration and its change during the reaction. By integrating this equation Ooshima *et al.* obtained an empirical relationship which suggests that the change in hydrolysis rate is due to the decline in specific activity of adsorbed enzyme. This in turn was explained by a change in the adsorption ratio of EGs and CBHs which reduces the synergism between them.

Kinetic studies of the hydrolysis of cellooligosaccharides (with DP up 8) by *T. reesei* CBHs (Nidetzky *et al.*, 1994*b*) revealed that the hydrolysis rate of the substrates by CBHI is initially very fast but drastically decreases within a short reaction time at rather low substrate conversion. For CBHII, a similar decrease in reaction rate was observed but the reaction stopped almost completely when more than 50% of the substrate was still not hydrolysed.

It is known that substrate structural parameters that affect the rate of enzymatic hydrolysis include crystallinity, accessibility, specific surface area and degree of polymerisation (Lee *et al.*, 1983). Fan *et al.* (1980) studied the effect of crystallinity and surface area of cellulose fibres on the enzymatic hydrolysis of cellulose and the changes of these structural features during the course of hydrolysis. They found that hydrolysis rate is more dependent on the crystallinity rather than surface area and that the latter is not a major limiting factor that slows hydrolysis in its later stages. Lee *et al.* (1983) observed that the crystallinity index of partially crystalline cellulose increases as the hydrolysis proceeds and the DP of highly ordered crystalline cellulose remains practically constant, whereas the DP of amorphous cellulose was found to be very significantly reduced.

It has also been demonstrated that the level of agitation greatly influences the activity of cellulolytic enzymes (Cavaco-Paulo, 1998*a*). Agitation enhances considerably the mass transfer through the boundary film at the surface of cellulose and consequently the time required to reach the apparent adsorption equilibrium is strongly reduced. The film at the liquid-solid interface may be up to 0.1 cm thick in systems without stirring and thus boundary film resistance may effectively decrease the rates of enzyme adsorption (Nidetzky *et al.*, 1994*a*).

The presence of lignin and hemicellulose in some cellulosic substrates is also reported as a limiting factor on cellulose hydrolysis since it acts as a physical barrier between the cellulolytic enzyme and its substrate (Abraham and Kurup, 1997; Gould, 1984). This has led to the necessity of developing different pretreatments (by physical, chemical and/or biological methods) to remove lignin and to reduce cellulose crystallinity when degrading lignocellulosic substrates.

Effect of surfactants on the hydrolysis of cellulosic substrates

The effect of surfactants on the hydrolysis of cellulosic substrates by cellulases has been investigated by several researchers (Duff *et al.*, 1995; Helle *et al.*, 1993; Kaar and Holtzaple, 1998; Ooshima *et al.*, 1986; Park *et al.*, 1992). They found that the rate of hydrolysis is influenced by surfactant type (nonionic, cationic and anionic) and concentration.

It was found that with nonionic surfactants, the hydrolysis rate was always improved and the type of nonionic surfactant and its concentration could influence the level of increase in the rate of enzymatic reaction (Helle *et al.*, 1993; Park *et al.*, 1992; Kaar and Holtzapple, 1998). It seems that nonionic surfactants act as enzyme stabilizers, preventing inactivation at the liquid-solid interface during hydrolysis and facilitating enzyme desorption from the substrate. It was found that the amount of free enzyme in the reaction mixture during hydrolysis was larger when surfactant was used than in the case of no surfactant (Park *et al.*, 1992). Another explanation for the increased hydrolysis rate of insoluble cellulose in the presence of nonionic surfactants is that the surfactant can adsorb to cellulose, thus lowering surface tension, improving wetting and thus making the cellulose more accessible to the enzymes (Helle *et al.*, 1992).

It has been shown, however, that cationic and anionic surfactants denature enzymes even at low concentrations (Ooshima *et al.*, 1986). The interactions between proteins and ionic amphiphiles have been widely studied. The results of these investigations indicate that electrostatic and or hydrophobic interactions occur between protein and surfactant molecules. In this case, the hydrophobic residues of the protein are exposed to allow the association with the surfactant and this usually results in conformational change of the native protein leading to its denaturation (Creagh *et al.*, 1993).

Cellulase synergism during hydrolysis

Synergism between the individual components of a cellulase system acting on insoluble cellulose adds further complexity to the study of the mechanism of cellulase action. Synergism studies have often been carried out using combinations of purified endoglucanases and exoglucanases.

In a review of synergism in cellulase systems, Woodward (1991) reported that synergism between cellulase enzyme components clearly depends on several factors, including the nature of the substrate and the concentration of components in a cellulase mixture. Moreover, synergism is not only successive action of endoglucanases and exoglucanases but is also related to the adsorption characteristics of these enzymes (Beldman *et al.*, 1988).

Henrissat *et al.* (1985) observed different types of synergism between the purified *T. reesei* cellulolytic enzymes in the degradation of cellulose. The synergism between CBHI and EGI or EGII depended on structural features of the substrate. Endo-exo cooperation was found with substrates of intermediate crystallinity whereas no synergism was recorded with microcrystalline cellulose or with CMC. On the other hand, synergism between CBHII and endoglucanases followed the pattern expected for an endo-exo cooperation. Endo-exo synergism can be well understood since endoglucanases provide free chain ends on the cellulose surface for the exoglucanases to act upon. Synergistic degradation of cellulose was also recorded with mixtures of CBHI and CBHII (exo-exo synergism).

Woodward *et al.* (1988*a*) studied the hydrolysis of Avicel with purified cellulase components from *T. reesei* at saturating and non-saturating concentrations. They found that maximum synergism was observed when the substrate was incubated with a non-saturating concentration of EGI or EGII with CBHI or CBHII. This finding could lead to a dramatic reduction in the requirement of

cellulase utilization. In later work, Woodward *et al.* (1988*b*) reported that that the cellulase concentration is a determinant factor for the degree of synergism in the hydrolysis of microcrystalline cellulose.

Walker *et al.* (1992) studied the synergism between the major *Thermomonospora fusca* cellulases (E2, E3 and E5) and *T. reesei* CBHI on cellulose fragmentation. The highest fragmentation activity was obtained with a mixture of E2, E3 and CBHI.

An interesting result was obtained by Ståhlberg *et al.* (1993) who found that the four major cellulases from *T. reesei* (CBHI, CBHII, EGI and EGIII), whether intact or truncated, all produced new reducing end groups on cellulose, and thus none of them has exclusively exocellulase activity. Both the rate of formation and the amount of reducing groups was considerably higher for EGs than for CBHs. EGIII was the most endo-active of the enzymes and CBHI the least.

Nidetzky *et al.* (1993) observed that the synergistic action of purified *T. reesei* cellulases decreased with increasing substrate concentration, depended strongly on the type of cellulose used, and was maximal on crystalline cellulose. In contrast, the activity of individual cellulases was highest on amorphous cellulose.

In further synergistic studies made with purified cellulases from *T. reesei* Nidetzky *et al.* (1994*c*) showed that combinations of CBHI-CBHII (exo-exo synergism) and EGIII-CBHI (endo-exo synergism) were the combinations having the greatest synergism, and that optimal ratios were a function of the total protein concentration.

Hoshino *et al.* (1997) also oberved synergism with certain combinations of exo-type cellulases, namely *T. reesei* CBHII, with other exo-type cellulases (*T. reesei* CBHI, *Irpex lactus* Ex-1 and *Aspergillus niger* Exo-A) during the saccharafication

of crystalline and amorphous cellulose. This synergism was verified by the production of cellobiose but not by the depolymerization of crystalline cellulose.

Kleman-Leyer *et al.* (1996) found that EGI and CBHII of *T. reesei* act synergistically to solubilise native cotton (producing soluble sugars) but not by increasing the rate of depolymerization.

Marx-Figini *et al.* (1997) studied the enzymatic degradation of cotton cellulose using separated endo- and exocellulases from *T. reesei* and concluded that an isolated endoglucanase (EGI) degrades cellulose without the presence of exoglucanase (CBHI), whereas the isolated exoglucanase is not able to attack cotton cellulose to a significant extent. It seems that exoglucanase can only act on cotton cellulose when it works in concert with endoglucanase.

Baker *et al.* (1998) investigated the hydrolysis of cellulose using ternary mixtures of purified cellulases from different origins to test their capacity of increasing the saccharification rate.

Samejima *et al.* (1998) used native bacterial cellulose and acid treated cellulose to investigate the synergistic effect between CBHI and EGII of *Trichoderma viride*. The rate of hydrolysis of the native bacterial cellulose increased drastically with the combination of the two enzymes, while no synergistic increase in the hydrolysis rate was observed with the acid treated cellulose. The synergism seems to be due to different assembly patterns of the microfibrils in the two cellulose samples, which was observed using electron microscopy.

Srisodsuk *et al.* (1998) showed in their study of the modes of action of CBHI and EGI *T. reesei* cellulases, on cotton and bacterial cellulose, that CBHI efficiently solubilises BMCC but does not decrease its DP. In contrast, the action of EGI resulted in a rapid decrease of DP, but less efficient overall solubilisation of the substrate. Isolated CBHI was practically inactive toward cotton, which has a high initial DP and a complex morphology. EGI rapidly reduced the DP of cotton

and slowly solubilised part of it. Working synergistically, EGI and CBHI solubilised cotton more rapidly and to a greater extent than EGI alone.

A highly synergistic effect between CBHI and EGI of *T. reesei* cellulases in simultaneous action was also observed with intact bacterial cellulose (BC), but this synergistic effect was rapidly reduced by acid pre-treatment of the cellulose. Distinct synergism was found upon sequential endo-exo treatment of BC but not in BMCC (Väljamäe *et al.*, 1999).

2.1.6 Analysis of cellulase components

Analytical techniques which enable the separation and purification of individual cellulases have clearly helped to advance the understanding of their (individual) modes of action. The discussion of synergism between cellulases (2.1.5) shows that it is also necessary to analyse, quantitatively as well qualitatively, the composition of natural cellulase complexes if the concerted action of their components is to be elucidated. Furthermore, methods which permit such analysis can be used to study the complex adsorption behaviour of mixtures via quantitative analysis of the supernatant before and after hydrolysis, and after desorption or dilution experiments. In the context of the present work, quantitative analysis of used process liquors is regarded as important for establishing whether the composition of recycled enzyme complexes needs to be adjusted in order to achieve the desired textile finishing effects.

Many authors have reported that purification of *Trichoderma reesei* cellulases is difficult due to the great number of isoforms (Medve *et al.*, 1998*b*). The differences between isoforms may be due to the differences in glycosylation (all *Trichoderma* cellulases, except EGIII, are glycoproteins). Another reason for confusion about the number of different cellulases has been that some catalytic domains may appear in culture filtrates separated from their CBDs. *Trichoderma* culture filtrates also contain other enzymes such as hemicellulases, amylases,

and proteases. This diversity, combined with the overall similarity of cellulases, makes their purification and quantitative analysis rather difficult.

The methods conventionally used are the measurements of the cellulase activities towards CMC and Avicel or FP, for endoglucanases and exoglucanases respectively. These methods are very useful when the aim is to get overall information about the cellulase activities of the sample in question, but if the aim is to get specific information about the single enzymes they fail, since these substrates are not selective for individual enzymes.

Radiotracer methods using differently labelled (^{3}H, ^{14}C) cellulases (Kyriacou *et al.*, 1989) have also been used but these only allow binding studies using binary mixtures.

Kolbe and Kubicek (1990) have used an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies to measure the concentration of the main cellulases from *T. reesei* (CBHI, CBHII and EGI) in commercial preparations, as well as in samples from laboratory fermentations. The method was found to be very sensitive (1-10 ng protein) with a coefficient of variability between 10 and 20% and was used to quantify the concentration of intact and truncated enzymes. This method has also been used for checking the degree of contamination of purified cellulase proteins and to estimate protein concentrations and the purity of enzyme preparations (Koivula, 1996).

By using genetic manipulation it is possible to eliminate particular cellulase activities by inactivation of the specific gene, or to increase the production of specific cellulases (Nevalainen *et al.*, 1991).
Protein and genetic engineering (Rubingh, 1997) can be used to tailor enzyme compositions to obtain more specific effects. These techniques have been applied to cellulases for finishing cotton fabrics (Cavaco-Paulo *et al.*, 1998*b*; Cavaco-Paulo *et al.*, 1999; Liu *et al.*, 2000) and for improving the thermal stability and reducing end-product inhibition of cellulases (Nevalainen *et al.*,

1991) and other enzymes. Other techniques such as surface residue mutation have been used to change the adsorption and activity properties of cellulases (Damude *et al.*, 1995; Mattinen *et al.*, 1997*a*; Wolfang and Wilson, 1999) and to improve their stability to denaturating agents such as anionic surfactants (Otzen *et al.*, 1999).

Genes of some enzymes can also be cloned in other organisms with the aim of producing enzymes for a particular purpose (Enari and Markkanen, 1977; Nevalainen *et al.*, 1991).

Chromatographic separation of crude cellulases of *T. reesei* has been used for purification and for qualitative and quantitative determinations of the enzyme components (Bisset, 1979; Bok *et al.*, 1998; Ellouz *et al.*, 1987; Fliess and Schügerl, 1983; Fontaine *et al.*, 1997; Hayne and Esterbauer, 1985; Kolbe and Kubicek, 1990; Medve *et al.*, 1994; Medve *et al.*, 1998*a*; Medve *et al.*, 1998*b*; Schmuck *et al.*, 1986; Woodward *et al.*, 1994; Yu *et al.*, 1993). Medve *et al.* (1998*b*) described a method for the quantification of cellulase mixtures that consists of a previous purification of the enzymes and then the use of reconstituted mixtures of the pure cellulases for hydrolysis of cellulose. Finally, the quantitative determination of enzyme concentration in the hydrolysate was performed by ion-exchange chromatography.

Nidetzky and Claeyssens (1994) used a similar method for quantitative determination of the major cellulases in reconstituted mixtures.

Adsorption studies with purified cellulases have provided valuable information on the adsorption characteristics of these components on cellulosic substrates. When crude cellulase mixtures are used for cellulose hydrolysis, however, it may be expected that the adsorption behaviour of the cellulase components will be different from those of the purified enzymes.

Chromatofocusing is a chromatographic technique for protein separation according to their isoelectric points (pI). Since the different cellulase components have different pIs (see Table 2.2, section 2.1.3), their separation can be achieved using this method. Indeed, this technique has been used to

purify, characterize and quantify cellulase components (Hayne and Esterbauer, 1985; Schmuck *et al.*, 1986; Woodward *et al.*, 1994; Yu *et al.*, 1993) and it is used in the present work to study the preferential adsorption of cellulolytic components on cotton cellulose (4.4.1).

2.2 Cellulase applications

2.2.1 Non-textile applications of cellulases

The great potential for cellulases in commercial applications provides the main driving force for research in this area. Cellulase applications have been discussed in several reviews (Béguin and Aubert, 1994; Bhat and Bhat, 1997; Henrissat, 1994; Ohmiya *et al.*, 1997, Teeri, 1997). Two main types of process are of particular importance: (i) partial hydrolysis of the cellulose substrate and (ii) the complete degradation of biomass to soluble sugars and their subsequent or simultaneous fermentation into solvents or fuels. Since fuels derived from lignocellulosics are not yet competitive with petrol or with products derived from starch hydrolysis, the economical feasibility of this process has been the subject of discussion. The application of cellulases in partial hydrolysis processes is well established and includes the major part of cellulase applications. In particular, cellulases are being used successfully in the textile and detergent industries and these applications are described in more detail in 2.2.2.

Considerable interest has been shown in the use of cellulases in the pulp and paper industries (Edgar *et al.*, 1998; Hata *et al.*, 1998; Jacobs-Young *et al.*, 1998; Kamaya, 1996; Ramos *et al.*, 1999*a*; Ryan *et al.*, 1998; Viikari *et al.*, 1998). The main purposes of this application are reduction of refining energy requirements, and enhancement of beatability and fibrillation of chemical pulps (Mansfield *et al.*, 1997). Moreover, the treatment of waste paper with enzymes can be used to assist the de-inking of newsprint for recycling which reduces the disposal costs and environmental impact of wastepaper.

Enzymatic treatment of other types of cellulosic wastes, which are produced in large amounts, either as municipal and industrial solid waste (Duff *et al.*, 1995; Itävara *et al.*, 1999; Moerkbak and Zimmermann, 1998; Moritz and Duff, 1996; Nikolov *et al.*, 2000; Van Wyk, 1998; Viesturs *et al.*, 1999) or as agricultural waste (Abraham and Kurup, 1997; Gould, 1984; Kaar and Holtzapple, 1998; Kaur *et al.*, 1998; Ramos *et al.*, 1999*b*) is also an important area. These cellulosic residues constitute an inexpensive carbohydrate source and can be converted to soluble sugars by using cellulases. Glucose can be used as medium for obtaining single-cell protein or for the production of ethanol and other alcohols.
Cellulases can also be used to produce cellobiose by removal of β-glucosidase activity during cellulose enzymatic hydrolysis (Homma *et al.*, 1993).

A further interesting application of cellulases, at least part of them, is the use of CBDs in fusion proteins, as tags for affinity purification, or immobilization in an inexpensive, chemically inert cellulosic matrix. This can enable their application in food and pharmaceutical industries (Greenwood *et al.*, 1994; Le *et al.*, 1994; Linder *et al.*, 1998; Ong *et al.*, 1989; Reinikainen *et al.*, 1997).
Recently CBDs have been also used for the immobilization of living cells (Doheny *et al.*, 1999; Wierzba *et al.*, 1995).

Other applications include the food, animal feed, brewing and wine making industries (Nevalainen *et al.*, 1991). In addition, cellulases have been used for the production of plant and fungal protoplasts, and in producing hybrid strains as well as in other genetic engineering experiments. More detailed descriptions of these applications are given by Bhat and Bhat (1997).

2.2.2 Cellulase applications in textile processing

Cellulosic textiles account for about 65% of total world textile fibre production (about 45 million tonnes per annum). This includes natural fibres such as cotton (50%), linen and ramie and regenerated cellulose fibres such as viscose, and lyocell. Thus, the textile industry represents a large potential market for cellulase applications.

In recent years there has been an increased interest in these enzymes for fibre modification. Enzymatic treatments have provided the creation of a variety of finishing effects (Kumar *et al.*, 1997), which can be obtained in moderate reaction conditions without using conventional chemicals that may produce toxic effluents.

Commercial cellulases for textile processing are mainly obtained from the fungi *Trichoderma reesei* (acid cellulase) and by *Humicola insolens* (neutral cellulase) (Cavaco-Paulo, 1998*a*; Klahorst *et al.*, 1994). Acid cellulases work in the pH range of 4.5-5.5 and their action is characterized of being highly aggressive. Neutral cellulases have pH optima around 6-8 and are known to be less aggressive, and to require longer treatment times (Klahorst *et al.*, 1994).

The most successful and more studied applications of cellulases in textile processing are biofinishing of cotton and the replacement of the stonewashing process to produce the fashionable aged appearance of denims. Other important benefits delivered during biofinishing are improvements in softness, hygroscopy and surface appearance by removing fuzz fibres and pills (Heine and Höcker, 1995).

Cellulases can be used to treat man-made cellulosic fabrics such as viscose rayon and lyocell (Cavaco-Paulo, 1998*b*; Kumar *et al.*, 1994). It was shown that cellulase could be applied on these substrates to improve softness and drapeability, and to reduce pilling tendency. Buschle-Diller *et al.* (1994) have also investigated the enzymatic hydrolysis of other cellulose fibres such as linen, ramie and viscose rayon.

The use of cellulases in forensic science investigations in which it may be possible to link fibres found at the scene of a crime with those from clothing worn by suspects has also been described. Cotton fibres dyed with reactive dyes (which can not be extracted with solvents) were digested using cellulases and the hydrolysis products analysed by thin layer chromatography (Rendle *et al.*, 1994).

A combination of cellulase enzymes with low temperature, oxygen plasma treatment was used to study their effect on the mechanical properties and dyeing behaviour of wool/cotton blend fabrics (Yoon *et al.*, 1996).

Recently, cellulases have been used, together with pectinases, in cotton scouring (Hartzell and Hsieh, 1998; Li and Hardin, 1997; Li and Hardin, 1998*a*, Li and Hardin, 1998*b*). It was demonstrated that enzymatic scouring of cotton can be a very effective step in the preparation of cotton for further chemical treatment and or dyeing (Li and Hardin, 1997).

Effects of fibre and fabric structure

The existence of different cellulosic textile materials, with different morphologies and structures, as well the specific effects desired on them, requires careful selection of enzyme compositions and tight control of process conditions (Kumar *et al.*, 1997).

The adsorption and hydrolysis mechanisms of cellulases on cotton cellulose are influenced by the parameters already mentioned in sections 2.1.4 and 2.1.5, respectively. The specific conditions used during textile processing, however, require some particular considerations. Research has provided both basic information and greater understanding of the mechanisms involved in enzymatic hydrolysis of cotton and this has led to improved cotton products and processing systems.

In textile applications, only partial hydrolysis of the fibres is desired and the enzymatic treatments must therefore be well controlled to minimise weight loss and damage to the mechanical properties of the fibres.
The activity of cellulase mixtures towards cotton cellulose is influenced by ionic strength and adsorbed ionic species as well by temperature and pH (Cavaco-Paulo, *et al.*, 1998*a*).

The physical form of cellulosic fibres clearly affects their accessibility, and the activities of cellulolytic enzymes towards them. The degree of swelling, orientation and crystallinity of different cellulosic fibres may be expected to lead to different rates of cellulolytic degradation. When the cellulose substrate is in the form of cotton or another cellulosic fibre fabric, the rate of cellulolytic attack is likely to depend on the hairiness of the fabric surface and the relative tightness or openness of the yarn twist and fabric construction.

Park *et al.* (1995) found that the effect of cellulase action on cotton depends on yarn linear density and fabric structure. It was observed for example that the weight loss on knitted fabrics is greater than for woven fabrics and fabrics constructed with finer yarns showed greater weight loss.

Fabric processing history also affects the rate of cellulolytic attack. For example, mercerising increases the accessibility of individual cotton fibres to cellulase (Buschle-Diller and Zeronian, 1994; Cavaco-Paulo *et al.*, 1998*a*), whereas raised fabrics are more accessible to enzymatic attack because they have more fuzz fibres on the surface. Mercerized fabrics show greater accessibility for enzyme attack, since they have more accessible fibre surface and a more open cellulose structure (Cavaco-Paulo *et al.*, 1998*a*; Cavaco-Paulo and Almeida, 1996).

Influence of dyes and surfactants

The presence of chemicals on textile material substrates, such as dyes, surfactants or other textile auxiliaries, can also influence the enzymatic treatment of cotton fabrics. This subject was studied by several authors (Buschle-Diller and Traore, 1998; Cavaco-Paulo *et al.* 1998*a*; Choe *et al.*, 1995; Koo *et al.*, 1994; Ueda *et al.*, 1995).

Koo *et al.* (1994) found that the rate of hydrolysis of cotton fabrics is reduced by both direct and reactive dyes adsorbed on the substrate. Vat dyes, however, did not show an inhibitory action. The results obtained by Cavaco-Paulo *et al.* (1998*a*) confirm these findings. These authors suggest that reactive dyes inhibit cellulose hydrolysis by reducing the number of sites available for CBH adsorption. This is probably because cellulose chains, carrying covalently bound dye molecules, cannot be easily accessed by the catalytic domains of CBHs which have active sites that are enclosed in tunnels buried inside the catalytic domains. Direct dyes probably inhibit cellulose hydrolysis by reducing the number of sites available for EG adsorption, since direct dye molecules possess large planar structures, which adsorb on hydrophobic edges of microfibrils and thus block many of the sites that would otherwise be available for EG adsorption. When vat dyes are oxidized and fully developed by "soaping" at the boil, they form relatively large, insoluble, crystalline aggregates that are firmly trapped inside the fibre, but are not bound at specific sites by strong physical forces. Consequently their presence does not significantly inhibit the hydrolysis process.

Buschle-Diller and Traore (1998) also examined the influence of direct and reactive dyes on the enzymatic hydrolysis of cotton. All reactive dyes studied decreased the hydrolysis rate and direct dyed fabrics showed a hydrolysis rate approximately equal to, or slightly higher than that for undyed fabrics. This latter result is contradictory to the findings of other authors (Cavaco-Paulo *et al.* 1998*a*; Koo *et al.* 1994).

The presence of cationic and anionic surfactants was shown to inhibit cellulase activity, whereas that of nonionic surfactants was not (Ueda *et al.*, 1994). Li and Hardin (1998*b*) found that nonionic surfactants during enzymatic scouring of cotton are essential for the enzymatic treatments to be effective. Nonionic surfactants are used to reduce the surface tension of cotton and assist the enzymes to penetrate the micropores.

Effects of agitation during processing

During textile processing, strong mechanical agitation is normally provided (rotating drum washers, winches and jets) (Cavaco-Paulo, 1998*a*), although in some applications relatively low agitation levels may be used. The effect of mechanical action during enzymatic treatments of cotton has been the subject of numerous studies since it has implications for delivering desired finishing effects (Cavaco-Paulo *et al.*, 1996*a*, Chong and Yip, 1994; Li and Hardin, 1998*b*; Liu *et al.*, 2000; Roussele and Howley, 1998).

It was observed that the degree of abrasion during enzymatic treatment of cotton influences the action of cellulolytic enzymes. The fabric weight loss increased with increasing fabric to liquor ratios at constant enzyme concentration (Chong and Yip, 1994).

Cavaco-Paulo *et al.* (1997) studied the effect of textile washing processes on cellulase-treated fabrics. They observed that the fibre surface did not change significantly after initial cellulase treatment, but after washing, the surface properties were completely different, depending on the type of cellulase treatment. It seems that subsequent mechanical action reveals the changes at the molecular level caused by prior cellulase activity.

Cavaco-Paulo (1998*a*) describes the role of mechanical agitation during cellulase application. It was observed that higher levels of mechanical agitation

enhance the endoglucanase activity by increasing the availability of sites for EG adsorption. This is explained by the fact that mechanical agitation causes more fibrillation and improves the access to fibre surfaces deep within the fabric structure. Moreover, in this situation the loose fibrils formed represented an increased and more exposed specific area for enzyme attack.

Further developments in textile finishing

Although the application of biofinishing is growing, it is still relatively limited compared to the market potential. A major obstacle to the broader use of biofinishing has been the limited availability of cellulase products that are adapted to the various types of textile processing equipment used commercially. Improvements in enzyme products are now being made better to fit various process needs of the textile industry.

Cavaco-Paulo *et al.* (1996*b*) have studied the finishing effects on cotton fabrics obtained with different cellulase compositions. They found that the characterization of enzyme mixtures is essential for the understanding and control of finishing effects and that characterization should be made under conditions that simulate real conditions of processing. The same authors (Cavaco-Paulo *et al.*, 1996*a*) observed that the endo activity of cellulases during cotton processing should be carefully controlled because it leads to high strength losses that are not desired in most textile applications. They also studied the effect of treatment time on cotton hydrolysis and found that short treatments keep the changes at the fibre surface while long ones affect the internal structure of the fibre.

Heikinheimo *et al.* (1998) used purified *Trichoderma reesei* cellulases to treat cotton. EGI and EGII had different effects to CBHI on cotton fabrics. Endoglucanases caused more strength loss than CBHI but also had positive effects on the fabric bending behaviour and pilling properties. The two

endoglucanases tested differed in their action. At low hydrolysis levels practically no strength loss was obtained with these endoglucanases whereas at higher dosages EGII caused significantly higher strength losses compared with EGI. Reduced pilling was, however, also obtained with these EGs at low enzyme dosages with practically no strength loss.

Liu *et al.* (2000) used different cellulase compositions to study the impact of processing conditions on biofinishing of cotton fabrics. The cellulase mixtures showed similar behaviour with regard to pH and temperature variations but their sensitivity to liquor ratio and mechanical agitation differred significantly. The authors therefore suggested that, for optimal biofinishing performance, an enzyme product should be selected on the basis of cellulase composition, existing equipment and fabric type.
Understanding the impact of each individual cellulase enzyme on the properties of different types of fabrics enables the development of novel optimised enzyme mixtures for the textile industry.

Cavaco-Paulo *et al.* (1999) have examined the possibility of using catalytically inactive cellulase proteins to bind to cotton fabrics and therefore create new textile applications. They found that when family II CBDs (CBD of CenA from *Cellulomonas fimi*) were bound to the fibres, the affinity of cotton fabrics for dyes increased, especially for acid dyes, which have a higher affinity for proteins. Ironing the fabrics further increased dye affinity, which was probably due to the exposure of ionic groups on the protein surface after heat denaturation. Wash fastness of these dyeings was worse than might have been expected for the acid dyes used, because of desorption of the family II CBDs in presence of surfactants and under the pH and mechanical agitation level used during the washing process. This approach opens new perspectives for delivering new benefits on cotton such as softness from the wash (Jones, 2000).

Some attempts have been made to determine adsorption and kinetic parameters during enzymatic hydrolysis of cotton and other cellulosic fibres

(Cavaco-Paulo and Almeida, 1996; Cavaco-Paulo *et al.*, 1998*a;* Cortez, 1999; Nidetzky *et al.*, 1994*c*). In these studies the authors assume a Langmuir adsorption isotherm and use a kinetic model analogous to the Michaelis-Menten approach (Nidetzky *et al.*, 1994*c*). The use of these models is, however, an over simplification of the complex heterogeneous system because the Langmuir model applies to the adsorption of single molecular species, whereas the cellulases in these studies were mixtures.

The Michaelis-Menten model was developed for a single enzyme type acting on a soluble substrate and does not account for the synergism present in many cellulase systems. Furthermore, the enzymatic hydrolysis of solid substrates may involve diffusion and boundary layer effects that are not accounted for in these models, unless the stirring rate is high enough to eliminate them.

Another objection to the application of these models to real textile processing systems, is that when adsorption and kinetics are studied at the optimum hydrolysis temperature, the extent of reaction occurring during the experiments may be sufficient to change the surface structure of the substrate. In this case the cellulase adsorption characteristics of the substrate would be varying, and this would invalidate the approach.

Nevertheless this approach provides useful comparative data for predicting the effects of different processing regimes (e.g. different agitation rates) or machine types (Cortez, 2000) on a given fabric, or the effects on different fabric or fibre types in a given process.

More usually the parameters used to measure the effect of the enzymatic treatments on textiles are tensile strength, weight loss, pilling, fabric hand, copper number, moisture regain and colour change (in the case of dyed fabrics). With the exception of copper number, however, these parameters do not provide mechanistic information, and therefore contribute little to the design of new, more predictable textile processing regimes.

Enzymatic "stonewashing"

The aged look of denims is obtained by non homogeneous removal of dye (usually indigo) through the cooperative action of cellulases and mechanical factors such as beating and friction (Cavaco-Paulo *et al.*, 1998*b*).
During the enzymatic treatment of denims, the removed indigo dye may redeposit on the white areas of denim fabric and diminish the contrast between the dyed and undyed regions of the fibre. This reduces the desired effect, and it is therefore important to control the level of backstaining in the process. The study of backstaining has received attention from numerous investigators (Andreaus *et al.*, 2000; Campos *et al.*, 2000; Cavaco-Paulo *et al.*, 1998*b*; Gusakov *et al.*, 1998; Gusakov *et al.*, 2000; Yoon *et al.*, 2000) and many attempts have been made to minimize this problem.

It has been reported that the level of backstaining is lower when neutral cellulases are employed instead of acid cellulases, but the causes of this phenomenon remain unclear (Cavaco-Paulo *et al.*, 1998*b*; Yoon *et al.*, 2000). These authors (Cavaco-Paulo *et al.*, 1998*b*) analysed independently the effects of several factors potentially related to indigo backstaining. These include different species present on the undyed cotton fibre surface, liquor pH and the type of cellulase used. The results obtained seem to confirm that backstaining is caused by the adsorption of indigo/cellulase complex onto the cotton substrate (Cavaco-Paulo *et al.*, 1998*b*). The same authors also postulated that reducing end groups in the fabric surface, produced during the enzymatic treatment, might induce indigo reduction (and hence re-dyeing), since the reduction of indigo by glucose at pH 5 and 50 °C does happen to a small extent. Etters (1995) has, however, reported that the reduction of indigo at pH 5-7 is not very effective and the reduced dye shows low affinity.
Indigo backstaining is reduced by some surfactants and desorption of cellulases from cellulose fibres is enhanced by a wide range of surfactants. This tends to support the view that at least some of the finely dispersed indigo in the aqueous phase is bound to enzyme proteins. Cavaco-Paulo *et al.* (1998*b*) also concluded

that pH is not the major factor in preventing backstaining and suggested that an enzyme that has very little affinity for indigo will minimise the problem.
Following this finding, Campos *et al.* (2000) studied the interactions of cellulase enzymes from different fungal origins (*T. reesei* and *H. insolens*) with insoluble indigo in the absence of cotton cellulose. They found that acid cellulases (*T. reesei*) have higher affinity for indigo dye than neutral cellulases (*H. insolens*). This seems to be due to the presence of more nonpolar amino acid residues on neutral cellulases and more neutral amino acid residues in acid cellulases. The particle size of insoluble indigo was also examined by incubation of indigo with the different cellulase types and different protein concentrations. Particle size measurements of indigo-isopeptide complexes indicate that some amino acid residues may promote agglomeration. *T. reesei* cellulases also showed preferential adsorption for indigo-dyed fabrics over undyed fabrics.

To avoid backstaining, denim processors are adding antiredeposition chemicals during the enzyme washing step and or adding a mild bleaching agent or stain release agent during the rinsing process (Yoon *et al.*, 2000). The same authors used a protease during rinsing, or at the end of the cellulase washing step, to degrade cellulase proteins and therefore prevent them from binding indigo back onto the denim surface. This gave a reduction of backstaining and improved the contrast between dyed and undyed regions.

In more recent work, Andreaus *et al.* (2000) concluded that reduction of indigo staining, in the presence of cellulases, can be achieved by "solubilisation" of indigo by soluble cellulases. They found that the indigo staining levels decreased with increasing fungal cellulase concentration. Removal of the CBD from cellulases from fungal and bacterial origin showed lower levels of indigo staining compared with the intact enzyme. This finding is of interest for tailoring new cellulases for enzymatic washing.

2.3 Possibilities for cellulase recycling

2.3.1 Recovery of enzymes from the liquor and the substrate

Enzymes are potential catalysts for a wide range of large-scale applications but the high cost of cellulase enzymes has been cited as a limiting factor for their application on a large scale (Kumakura, 1997; Moniruzzaman *et al.*, 1997). Consequently, attention has been devoted to designing efficient reaction processes, sometimes with a view to enzyme recycling.
For example, economic evaluation of the enzymatic hydrolysis of phenol-pretreated wheat straw (Zacchi *et al.*, 1988), revealed that enzyme recovery appears to be necessary for the economic feasibility of the process. Similarly, Nguyen and Saddler (1991), in their work on the technical and economic evaluation of an enzymatic biomass conversion process, concluded that enzyme recycling of the hydrolysate can reduce the net enzyme requirement and thus lower the costs. Thus it is not surprising that considerable attention has been focused on the use of recovered enzymes for the hydrolysis of cellulosic substrates.

During the course of hydrolysis of cellulosic materials, the cellulases are distributed between the solid substrate and the supernatant. Cellulases can therefore be recovered from either phase of the reaction. The simplest method for cellulase recovery is readsorption of enzymes present in the supernatant onto fresh substrate. Cellulase recovery would, however, be incomplete, since a portion of the enzymes would remain adsorbed to the original substrate. Many agents have been used to elute cellulase activity from various substrates, such as surfactants (Otter *et al.*, 1989; Rao *et al.*, 1983), glycerol (Desphande and Ericksson, 1984; Otter *et al.*, 1989), alkali (Otter *et al.*, 1989) and phosphate and acetate buffer of varying pH (Desphande and Ericksson, 1984; Sinitsyn *et al.*, 1983). The best results were obtained in a study which used a single extraction procedure, with alkali and Tween-80, to recover adsorbed cellobiohydrolase

from Avicel (Otter *et al.*, 1989). In this case, 65% of the adsorbed Avicelase activity was recovered.

Eklund *et al.* (1992) studied the recovery of cellulases by adsorption onto steam-pretreated willow using various temperatures and pHs. They found that the recovered activity was greater below 40 °C and pH 7.5 and that it was possible to achieve 58% of total activity from the hydrolysate and solid residue.

An investigation into the use of enzyme recycling on degrading wood chips with *Trichoderma* cellulases (Ramos *et al.*, 1993*b*) showed that most of the cellulase activity was associated with the unhydrolysed residue (peroxide-treated fraction derived from stream-exploded *Eucalyptus viminalis* chips). The enzyme mixture used to obtain complete hydrolysis of the substrate was successfully recycled for five consecutive treatments. The efficiency of cellulase hydrolysis gradually decreased with each subsequent treatment.

Ramos and Saddler (1994) showed that it is possible to recover and recycle cellulases for hydrolysing seven consecutive batches of steam-exploded *Eucalyptus viminalis*, without any requirement for further addition of cellulases after the first hydrolysis step. Considerable loss of protein and enzyme activity was observed throughout the experiment.

Moniruzzaman *et al.* (1997) demonstrated that it was possible to hydrolyse corn fibre by repeated recovery and reuse of a portion of the enzyme preparation, with addition of a small portion of fresh enzyme in each subsequent recycling step. They observed that most of original activity could be recovered in the first step, but a gradual decrease in the recovered enzyme activity was observed in the later stages of recycling, probably owing to processes, such as thermal and mechanical inactivation.

Enzyme immobilization is often used to improve enzyme stability and provide the easiest method for its recovery and reuse (Busto *et al.*, 1997; Chim-anage *et*

al., 1986; Kumakura, 1997; Taniguchi *et al.*, 1992; Triantafyllou *et al.*, 1997; Woodward and Zachry, 1982). Although cellulolytic enzymes have been immobilized quite successfully by covalent bonding on water-insoluble supports, such enzymes cannot be used for the efficient and complete hydrolysis of insoluble cellulosic materials.

Methods for cellulase recovery and concentration have also been reported as downstream processing. Avelino *et al.* (1999) developed a method for cellulase recovery and concentration by a two step process involving precipitation followed by flotation.

Lee *et al.* (1995) evaluated three recycling strategies to determine their efficiencies over five successive rounds of hydrolysis of lignocellulosic substrates. The recycling strategies were: recycling of enzymes adsorbed to the residual substrate, recycling of enzymes adsorbed to the residual substrate and present in the supernatant and recycling of enzymes in the noncellulosic residue and the supernatant. They observed that when cellulases were recovered from the residual substrate after partial hydrolysis, the recovered cellulase activity toward the mixture of fresh and residual substrate decreased after each recycling step. When the cellulases in the supernatant were also recycled, up to 20% more activity could be recovered. In both of these cases, the recovered activities did not correspond to the activities expected from the amount of cellulase protein recovered during recycling. The best recovery was achieved when the cellulases were recycled from both the residue and the supernatant after complete hydrolysis of the substrate. In this case, all of the originally added cellulase activity could be recovered for four consecutive hydrolysis rounds. It was also shown that when recycling enzymes adsorbed on the residual substrate, cellulases were quickly partitioned between the fresh and residual substrates.

When repeated recycling of cellulases present in supernatants is carried out, the hydrolysis products (mainly glucose and cellobiose) accumulate in the

reaction mixture (Ramos *et al.*, 1993*b*). The inhibition of cellulase components by the end products is well known and this constitutes a major problem in recycling. It is therefore necessary to provide a method for separating cellulases from end products to improve the activity of the recycled enzymes.
Vallander and Ericksson (1987) found, in their work on enzyme recycling in saccharification of lignocellulosic materials, that the hydrolysis process became more efficient if end-product inhibition was limited by intermittent hydrolysate removal (two-step hydrolysis) and by short contact times between enzyme-containing hydrolysate and new substrate. Improved sugar yields were also obtained by residue washing.
The use of ultrafiltration (UF) techniques, for the removal of sugars produced during hydrolysis and for recovery and concentration of cellulases has been suggested by several groups of workers (Frenz and Bleschek, 1990; Gregg and Saddler, 1996; Ishiahara *et al.*, 1991; Ramos *et al.*, 1993*b*; Tanaka *et al.*, 1988). UF membranes with a specific molecular weight cut-off (MWCO) can be used to retain the enzymes with high molecular weight and allow the hydrolysis products with lower molecular weights to pass through.

An alternative to UF, for separation of cellulases from end products, is the selective adsorption of cellulases from the liquor. Emert (1980) used fresh cellulosic substrate to adsorb cellulases; the cellulosic substrate then became part of the feedstock for a further saccharification process.
Fujishima *et al.* (1988) have patented the use of chitin and chitosan derivatives for recovering cellulases from saccharification processes. At acid pH the chitin/chitosan is soluble and strongly adsorbs cellulases. On raising the pH the chitin/chitosan, with adsorbed cellulase, is precipitated and can be recovered by filtration. The recovered material can then be dissolved in an acidic buffer for a further saccharification process. Recovery of 60-92% of the cellulases remaining in solution is claimed.
Woodward and Ridge (1989) have patented the use of commercially available Kiesselguhr (DEAE-Macrosorb) for separating cellulases from sugars after saccharification of cellulose. The cellulases were adsorbed onto the Macrosorb

at pH 5-8 which was then separated by decantation or filtration. The cellulases were released from the adsorbent at pH 2.5-4.0.

Other techniques have been proposed to alleviate end-product inhibition such as carrying out the hydrolysis in a two-phase system (Mandenius *et al.*, 1988), which permits the enzymes and the substrate be partitioned to one phase and the products to be extracted into a second phase. In addition, two-phase systems allow recycling of the enzymes.

It should be noted that no reference has been found to work on the recycling of cellulases after their use in textile processing.

2.3.2 Evaluation of the main problems for cellulase recovery and recycling in textile processing

In all recycling studies described above, a decrease in the cellulase activity with successive recycling steps is mentioned. The factors that may contribute to this are:

(i) loss of enzyme protein on the substrate and filtration membranes;

(ii) loss of particular cellulase components leading to changes in the synergism between EGs and CBHs;

(iii) end-product inhibition;

(iv) denaturating of enzyme protein by thermal and mechanical ageing.

In the context of the present work, each of these types of activity loss need to be considered. In particular, changes in the composition of cellulase complexes, caused by differences in the reversibility of binding of individual components to the textile substrate, are considered to be important. This is because cellulase finishing on textiles requires small but reproducible changes to be made in the physical properties of the substrate surface, and this requires constant composition of the enzyme complex (Cavaco-Paulo *et al.*, 1996*b*).
Furthermore, since textile processing is often carried out on dyed substrates in the presence of surfactants, the effects of interactions between cellulases and dyes and surfactants on cellulase recovery and recycling also need to be considered.

2.3.2.1 Inhibition of cellulases by end products and other compounds

It is well known that most cellulases are inhibited by cellobiose or glucose or by both products (Gusakov and Sinitsyn, 1992, Holtzapalle *et al.*, 1990; Lee and Fan, 1983). It has been reported that two types of inhibition, competitive and non-competitive may be observed. In non-competitive inhibition, the inhibitor affects both the maximum reaction rate and the effective Michaelis constant (K_m) whereas in competitive inhibition only K_m is affected.

It has been reported (Vallander and Ericksson, 1987) that cellulases are not inhibited until the sugar concentration reaches about 6%. This would imply that end-product inhibition should not be a problem in textile processing, even after several recycling steps, since substrate weight loss is generally not more than about 1% and final sugar concentrations would be of the order of 0.1% per use cycle. Nevertheless this is a finding that needs to be checked for textile processing applications (see 4.2).

Holtzapple *et al.* (1990) studied the inhibition of *T. reesei* cellulases by sugars and solvents. They observed that glucose and cellobiose were non-competitive

inhibitors of the cellulase activity and they suggested that cellulase enzymes have a regulatory (alosteric) site where the inhibitors bind. It is known, however, that the active site of the enzyme is structured to accept sugar polymers, and it is likely that sugar monomers and dimers would also bind to the active site.

The enzyme β-glucosidase (cellobiase) is essential in cellulose hydrolysis for the conversion of cellobiose to glucose, because cellobiose is a strong inhibitor of other cellulase components (Calsavara *et al.*, 1999; Dekker, 1986). The action of β-glucosidase on cellobiose also shows substrate inhibition. Cellobiase suffers competitive or mixed inhibition by glucose (Holtzapple *et al.*, 1990). Since cellobiose is water soluble, cellobiase can be used for enhancement of cellulose hydrolysis, in the form of immobilized enzyme. This can be achieved using a column adjacent to the main reactor, through which the supernatant is circulated.

T. reesei cellulase preparations are relatively deficient in β-glucosidase activity. These preparations can be supplemented with exogenous β-glucosidase preparations derived from other microorganisms to increase the rate and extent of cellulose saccharification (Dekker, 1986).

Kaya *et al.* (1999) investigated the effect of dissolved lignin and related compounds on the enzymatic hydrolysis of CMC. They found that low concentrations of lignin degradation products (vanillin, vanillic acid, acetovanillone, protocatechuic acid and guaiacol) tended to improve hydrolytic activity, but at higher concentrations some of these compounds caused cellulase inhibition.

2.3.2.2 Interactions of cellulases with surfactants

Cellulases have attracted interest in the detergent industry because of their ability to soften fibrillated cotton and brighten faded coloured garments by

removing surface fibrils. It is not, however, an easy task to obtain a cellulase that functions efficiently under fabric washing conditions. Insight into the interactions between proteins and detergents is essential. Detergent products typically contain both nonionic and anionic surfactants and are formulated to give strongly alkaline solutions. Nonionic or zwitteronic surfactants generally stabilize proteins and are often used to solubilise proteins that require a hydrophobic environment (eg. membrane proteins). In contrast, ionic surfactants, particularly anionic surfactants, partially or completely denature proteins.

Some proteins have specific surfactant binding sites in the native state. For example, serum albumin has 10-11 such sites for single surfactant anions, so the protein is actually stabilized against urea by very low concentrations of anionic surfactants (Otzen *et al.*, 1999).

Otzen *et al.* (1999) studied the unfolding of endoglucanase EGV from *H. insolens* in presence of denaturant (guanidinium chloride, GdmCl) and surfactant (linear alkyl benzene sulfonates - LAS). They observed that the protein unfolds in the presence of GdmCl according to a simple two-state model. EGV unfolds in very low concentrations of C12-LAS (1-4m*M*). The data suggest that the introduction of positive charges, or removal of negative charges, greatly increases surfactant sensitivity, while interactions with the hydrophobic detergent tail contribute to a smaller extent.

Anionic surfactants deactivate proteins at very low concentrations, down to 10 μM, in the case of C12-LAS. Therefore, their mode of action must involve strong interactions between the detergent molecule and the protein molecule, with preferential binding to the denatured state.

2.3.2.3 Mechanical and thermal deactivation of cellulases

Since cellulases have a wide range of industrial applications, it is extremely important to understand the stability of these enzymes under application

conditions. Furthermore, better understanding of the mechanisms of deactivation may lead to the development of more stable and less easily deactivated enzymes.

Native proteins are stabilized by a combination of hydrogen bonding, van der Waals forces, electrostatic and hydrophobic interactions and in some cases by cross-linking (e.g., disulphide bonds), metal complexing and cofactor binding. Heat, pressure, extremes of pH, urea, and shearing forces can disturb the delicate structure of proteins and can therefore denature enzymes. In the context of protein denaturation, reversible and irreversible changes can be distinguished. Reversibility can be described as the spontaneous reversion to the native state upon removal of the denaturating agent(s).

Ganesh *et al.* (2000) investigated cellulase deactivation in a stirred reactor at different rates of agitation. They varied enzyme and buffer concentrations, buffer systems and pHs and the presence of a gas-liquid interface. It was found that on increasing agitation the deactivation of cellulases was enhanced due to the increase of shear stress. When surface aeration was prevented, however, the deactivation effect was reduced. At higher pHs, cellulases showed greater deactivation but this was mainly due to the high pH environment rather than to agitation.

The amphipathic nature of proteins, as a result of a mixture of polar and nonpolar side chains, causes protein molecules to be concentrated at interfaces. Protein denaturation at hydrophobic interfaces normally follows a mechanism by which protein molecules unfold to expose hydrophobic regions to the interface, and then the unfolded molecules aggregate through interaction between their hydrophobic regions. The aggregates eventually desorb from the surface and precipitate.
Enzyme inactivation kinetics typically follows first-order behaviour:

$$\ln\left(\frac{A}{A_0}\right) = -k_{obsd}t$$

where A_0 is the enzymatic activity at t = 0 and k_{obsd} is the observed inactivation rate constant.

The inactivation process is known to involve a number of reversible (dissociation and denaturation) as well irreversible (decomposition, aggregation, and coagulation) reactions. These reactions can combine to form a wide variety of reaction pathways, which can potentially demonstrate complex inactivation kinetics (Lencki *et al.*, 1992).

Furcht and Silla (1990) studied cellulase deactivation during enzymatic hydrolysis of cellulose in ball mill and attrition mill reactors. They examined the effects of different milling media and particle sizes and the presence of an air-liquid interface in the reactor. It was shown that the enzyme activity after six hours milling ranged from 1 to 85% of the starting activity depending on milling media, presence of interface and treatment time.

The deactivation of cellulase in high shear fields was also studied by Kaya *et al.* (1996). The activity of cellulase decreased with increasing shear rate and elapsed mixing time. Deactivation occurred very quickly (after 10 minutes of mixing). It was concluded that high shear or prolonged exposure to low shear, changes the molecular structures of the enzymes causing denaturation.

Reese (1980) and Kim *et al.*, (1982) have also reported the deactivation of cellulases by shaking, and the effect of the air-liquid interface. It was demonstrated that the cellulase deactivation due to the interfacial effect combined with shear, was far more severe and extensive than that due to the shear effect alone. It was proposed that the surface tension at the interface deactivates by unfolding the enzyme. Both increased cellulase concentration, and the addition of nonionic surfactant reduced the degree of deactivation. By using sufficient nonionic surfactant the cellulase deactivation could be

completely prevented, and the usefulness of the enzyme was therefore prolonged. The stabilizing effect of surfactant was attributed to the reduction in surface excess of cellulase (Kim *et al.*, 1982). Other effective protective agents against cellulase inactivation are fluorocarbons and high molecular weight polyethylene glycols. Some compounds, e.g., thymol, were found to increase the rate of inactivation caused by shaking (Reese, 1980).

Baker *et al.* (1992) studied the thermal denaturation (from 22 to 70 °C) of purified *T. reesei* cellulases (CBHI, CBHII, EGI and EGII) by classical activity profiles, differential scanning calorimetry (DSC) and thermal scanning fluorescence emission spectrometry. These studies showed that EGII is more thermostable (by 10-11 °C) than the other three enzymes. The DSC results suggested that at least two transitions are involved in the unfolding of each cellulase component, the first of which is likely to be the one correlated with activity loss.

The stabilization of cellulase enzymes has been reported by several authors (Abdel-Naby, 1999; Bilen and Bakir, 1998; Busto *et al.*, 1997).
Stabilization against thermal inactivation can be performed in several ways such as the use of additives (soil humates, polyethylene glycols, glycerine, betaines and related compounds) or by cross-linking the enzyme to a water-insoluble carrier, covalent coupling to natural or synthetic polymers, or by entrapment in gels. Many proteins containing carbohydrate residues exhibit increased stability towards heat and storage, which in many cases seems to be due to the carbohydrate part of the molecule. Most glycoproteins exhibit high water stability, and thus it was considered that stabilized water-soluble enzymes might be obtained through covalent attachment to carbohydrates. Hydrogen bonding between the polysaccharide and the protein surface and intra- as well intermolecular crosslinks between the protein and polysaccharides have been suggested as causes of thermal stabilization of the synthetic glycoproteins (Abdel-Naby, 1999).

Bilen and Bakir (1998) studied the thermal stability of cellulases and found, contrary to Baker *et al.* (1992) that cellobiohydrolase showed the highest heat stability and β-glucosidase the lowest. Thermal inactivation starts in the range of 55 to 65 °C. Some crosslinkers were tested in order to study their ability to enhance the thermal stability of cellulases. Only dimethyl suberimidate was found to slightly increase the thermal stability of cellulases.

2.4 Conclusions from the literature – objectives

This review has shown that there is a large and rapidly growing body of literature on the structure, analysis, adsorption and mode of action of cellulases, and that the use of genetic engineering is making it possible to tailor cellulase complexes for specific purposes, including applications in textile finishing.

The recycling of cellulases has been studied in some industrial applications in order to reduce costs and make their use more economically attractive. In textile processing, however, all the attention has been focused on developing new effects, better controlled processes, and improved cellulase compositions. No attempts to recycle cellulases after use in textile processing have been found reported in the literature and a study of the possibilities in this area therefore seems to be novel and timely.

Potential barriers to cellulase recycling such as end-product inhibition, thermal and mechanical deactivation, and complex formation with surfactants and dyestuffs have been widely reported, and the evaluation of these problems in context will therefore form an important part of this work.

The change in composition of cellulase complexes remaining in solution after processing, caused by differential adsorption effects between cellulase components, is a potentially more serious problem for recycling after textile processing, than in other applications such as saccharification of biomass.

A fundamental study of the adsorption and desorption behaviour of various cellulase components on cotton cellulose therefore forms an important part of

this work. The need to quantify the composition of cellulase complexes before and after adsorption required an appropriate analytical method to be established. Indications from the literature suggested that Fast Protein Liquid Chromatography (FPLC) would be most suitable and it became a further objective of the work to apply this technique to the analysis of cellulases used in cotton processing.

3. EXPERIMENTAL

3.1 Materials

3.1.1 Chemicals

General chemicals

The following analytical grade chemicals were used in this work: acetic acid (glacial), phosphoric acid (85%), ethanol (absolute), Folin-Ciocalteus's phenol reagent (2 *N*), sodium hydroxide pellets, sodium carbonate, Coomassie Brilliant Blue, neocuproin-hydrochloride, copper (II) sulphate-pentahydrate, sodium tartrate 4-hydrate, sodium chloride, sodium dithionite.
Glucose, cellobiose and bovine serum albumin (BSA) were of "biochemistry use" purity. All chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (Sigma Chemical CO., St. Louis, MO, USA) and used without any further purification.
A kit for determination of glucose was purchased from Sigma (Sigma Diagnostic, St. Louis, MO, USA)

Chromatography chemicals

For the chromatography experiments all the chemicals, including water, were of HPLC grade and were purchased from Merck (Darmstadt, Germany) or Sigma (Sigma Chemical CO., St. Louis, MO, USA).
The eluent (Polybuffer™ 74) was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).
All buffers and solutions were prepared in water for HPLC and were filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Dassel, Germany) in a 1-L all glass filtration apparatus (Millipore, Bedford, MA, USA) before being used in the column.

Dyes

Two dyes were used for dyeing the cotton fabrics. A reactive dye (Remazol Brilliant Blue R, Ciba, Switzerland) and a vat dye (Brilliant Indigo 4B-D 150%, BASF, Ludwigshafenrhein, Germany). The chemical structures of the dyes are represented in Figure 3.1.

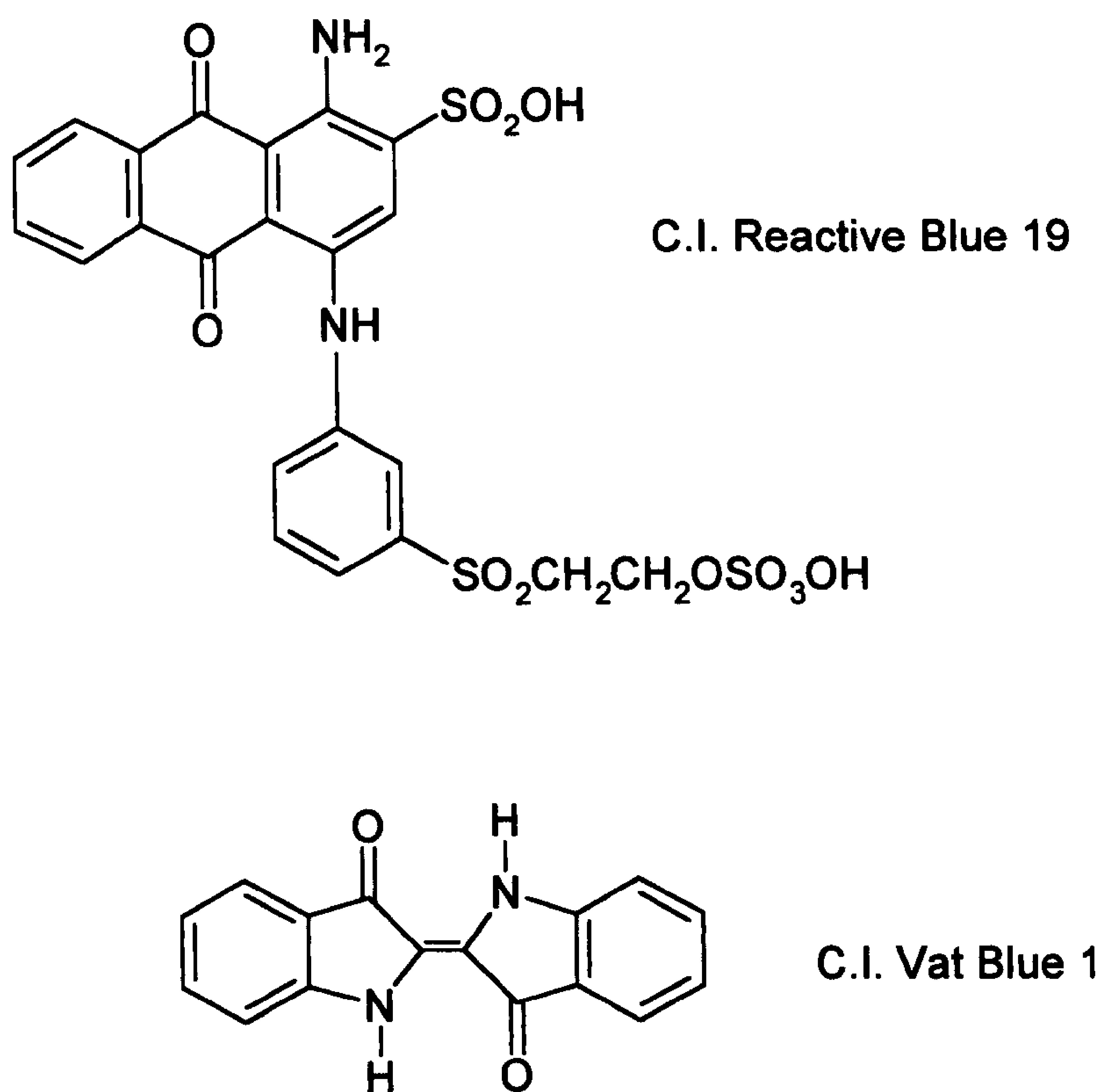


Figure 3.1 Chemical structures of the dyes: Remazol Brilliant Blue R (C.I. Reactive Blue 19) and indigo (C.I. Vat Blue 1).

Buffers

The buffer systems used in this work for the enzymatic reactions were acetate and phosphate buffers. They were prepared at 0.1 *M* concentration using acetic

acid and phosphoric acid, respectively. The pH was adjusted with sodium hydroxide solution (10 *M*) to achieve the values of pH 5.0 and pH 7.0, respectively.

Surfactants

The surfactants used in this work were an anionic surfactant (Lutensit A-LBN 50, sodium salt of C_{10-13} – alkylbenzesulphonic acid) and a nonionic surfactant (Lutensol A 7 N, alkylpolyethylene glycol made from a saturated, 100% linear C_{12-14} fatty alcohol with an average ethoxyl chain length of 7. The surfactants were purchased from BASF (Ludwigshafenrhein, Germany).

3.1.2 Substrates

When the enzyme activity towards cotton fabrics was measured, the substrate used was scoured and bleached 100% cotton poplin fabric having 60/32 ends/picks cm^{-1} and area density of $100gm^{-2}$. The cotton fabrics were used without any further treatment, or they were dyed following the procedures described below (3.2.14) and using the dyes mentioned above. For the desorption experiments with surfactants, the fabric was washed in 2 g/L sodium carbonate solution and rinsed in hot and cold distilled water to remove any residual surfactant.

For the filter paper (FP) activity assay, Whatman filter paper No. 1 (Whatman, Maidstone, UK) was used as the substrate.

The CMC activity (endoglucanase activity) was measured towards high viscosity carboxylmethylcellulose (sodium salt, degree of substitution less than 0.4) from BDH Chemicals, Ltd. (Poole, England).

Cellobiose was used as the substrate for β-glucosidase.

3.1.3 Enzymes

In this work a variety of cellulase compositions from different origins were used. The crude preparations were of fungal origin namely from *Trichoderma reesei* and from *Humicola insolens.* The *Trichoderma* cellulases were supplied by Röhm Enzyme Finland Oy (Rajamäki, Finland) and the *Humicola* cellulases were from Novo Nordisk (Bagsvaerd, Denmark).

Purified preparations of *Humicola insolens* EGV and its isolated core were obtained from Novo Nordisk and purified endoglucanase (CenA) and its isolated catalytic domain from the bacterium *Cellulomonas fimi* was kindly donated by Prof. Douglas Kilburn (University of British Columbia, Vancouver, Canada).

Approximate compositions of the cellulase preparations used in this work, as well some other characteristics are described in Table 3.1.

Table 3.1 Compositions and characteristics of the cellulase preparations used in this work.

Cellulase preparation	Gene deleted	Enzyme ratio (%)	Optimum pH	Optimum temperature	Supplier
Total crude (TC) Ecostone L 883042	None	CBHI (62), CBHII (20), EGI (10), EGII (8), minor EGs	4.5-5.5	45-50 °C	Röhm
CBH-rich CE 519/92	EGI, EGII	CBHI (75), CBHII (25)	4.5-5.5	45-50 °C	Röhm
EG-rich CE 523/92	CBHI, CBHII	EGI (50), EGII (50)	4.5-5.5	45-50 °C	Röhm
Total crude (TC) Denimax L	None	Information not available	7.0-7.5	55-60 °C	Novo

3.2 Experimental methods

3.2.1 Determination of soluble reducing sugars

Reducing sugars released into the liquor were measured by the method described by Cavaco-Paulo (Cavaco-Paulo, 1995).

Description of the method

This method depends on the reduction of the neocuproine-Cu (II) complex (yellow solution) to neocuproine-Cu (I) (orange solution) when heated with reducing sugars in alkaline media. The colour change can be monitored by reading the absorbance at 475 nm.

Preparation of neocuproine reagent

Copper (II) sulphate pentahydrate (0.2 g) and neocuproine chloride (0.4 g) were dissolved in distilled water to a final volume of 1000 mL.

Reducing sugars assay

0.05 mL of sample, 2 mL of sodium carbonate (2%, w/v) and 5 mL of neocruproine reagent were mixed in a test tube and then incubated during 5 minutes in a boiling water bath. After cooling, 10 mL of distilled water was added. The absorbance at 475 nm was measured against a reagent blank using an appropriate buffer in a UV-Vis spectrophotometer (Heλios γ Unicam, Cambridge, England). The determinations were done in duplicate or triplicate and the final value given is the arithmetic mean of those determinations. Glucose was used as the standard reducing sugar to construct the calibration curve shown in Figure 3.2. For each set of sample determinations glucose standards were also included, rather than relying solely on the calibration curve.

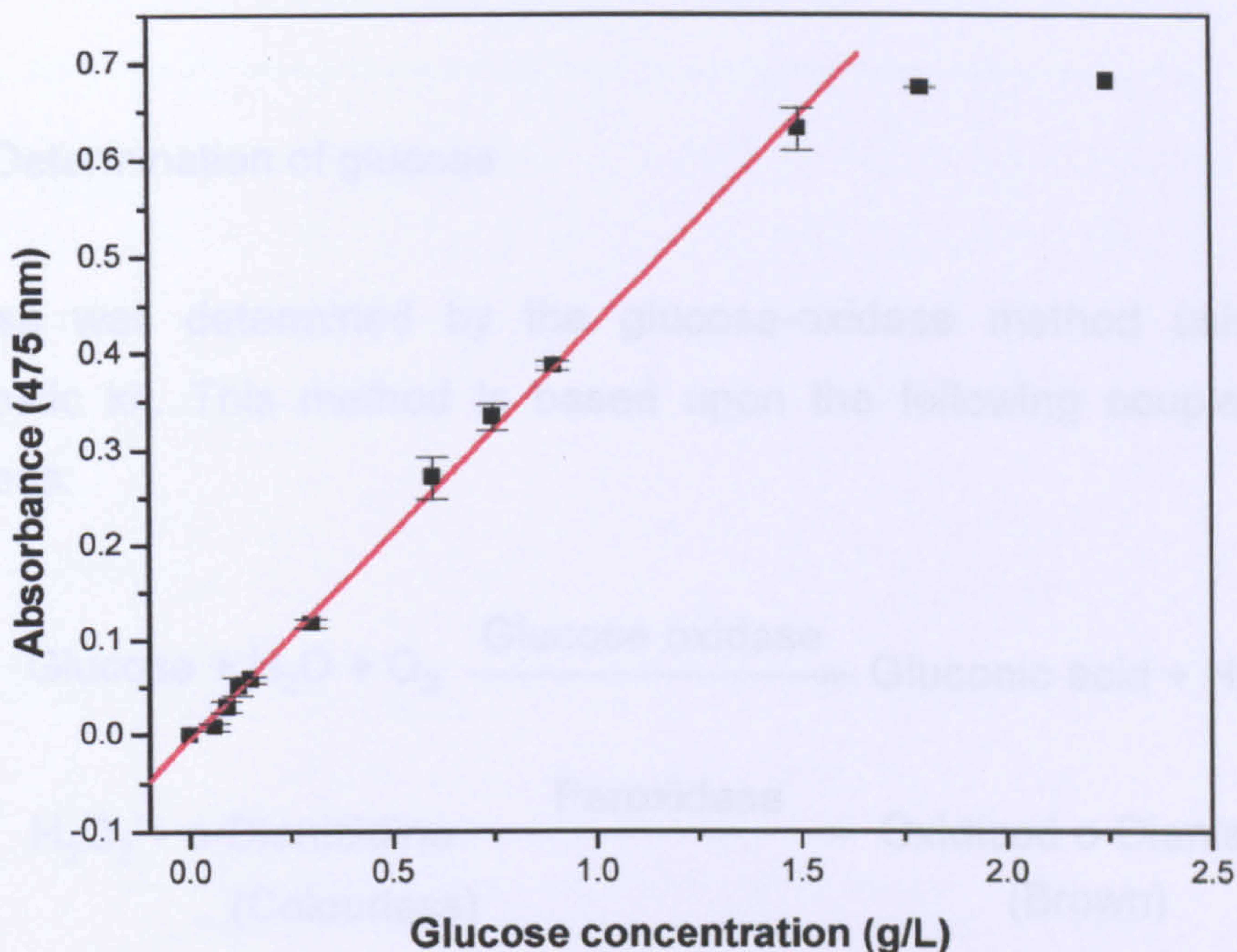


Figure 3.2 Calibration curve for measuring soluble reducing sugars by the neocuproine method, using glucose as standard sugar.

3.2.2 Determination of cotton reducing power

The reducing power of the end group content of fabric samples was determined by boiling 100 mg of cotton sample in a alkaline solution of neocuproine-Cu (II) complex as described above (3.2.1). The reducing power of cotton (mg glucose/g cellulose) was calculated using the following equation:

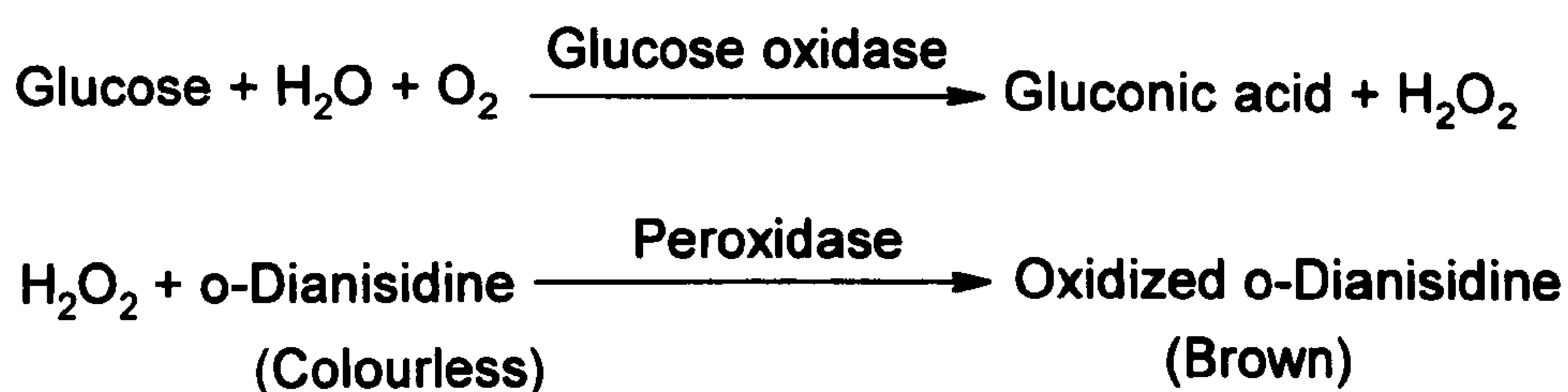
$$\text{Reducing power} = \frac{[\text{sugars}] \times V}{W}$$

where [sugars] is the concentration of reducing sugars measured in the sample analysed (mg/mL), V is the sample volume (0.05 mL) and W is the weight of

cellulose sample (0.1 g). Three determinations were made for each fabric sample and the final value is the arithmetic mean of those determinations.

3.2.3 Determination of glucose

Glucose was determined by the glucose-oxidase method using a Sigma diagnostic kit. This method is based upon the following coupled enzymatic reactions:



The experimental procedure was carried out by incubating 0.5 mL of sample (diluted 1:20) in a test tube with 5.0 mL of combined enzyme-colour reagent at 37 °C for 30±5 minutes in shaker bath at 125 rpm. After this period, the absorbance was read at 450 nm using water as reference. A glucose solution (1 g/L) diluted 1:20 was used as standard in all measurements for determination of glucose concentration in the samples. The glucose concentration of the samples was calculated using the following expression:

$$[\text{Glucose}](g/L) = \frac{A_{sample}}{A_{standard}}$$

where the A_{sample} and $A_{standard}$ are the absorbance values at 450 nm of the sample and glucose standard (1 g/L), respectively.

A calibration curve, using a range of glucose concentrations was made to determine the concentration range for which the absorbance vs concentration curve is linear (Figure 3.3). Two determinations were made for each sample and the final value is the arithmetic mean of those determinations.

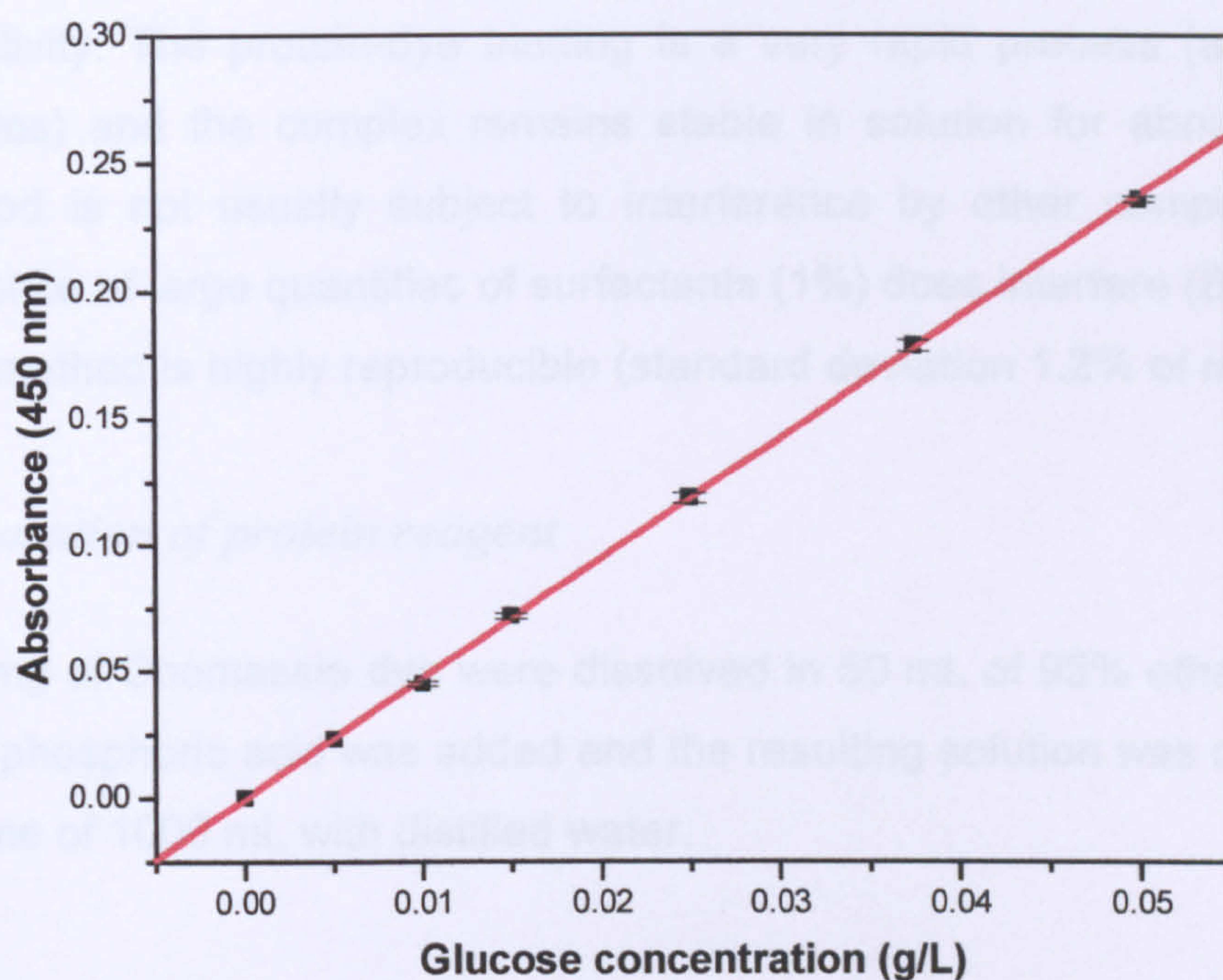


Figure 3.3 Calibration curve for glucose determination by the glucose-oxidase method.

3.2.4 Determination of total protein in solution

Total protein in the liquor was measured by the Bradford assay (Bradford, 1976).

Description of the method

The method is based on the observation that Coomassie Brilliant Blue G-250 exhibits a blue colour when it is bound to a protein. The binding of the dye to the protein causes a shift in the absorption maximum of the dye from 465 nm

(red) to 595 nm (blue). Consequently, the protein-dye complex can be measured spectrophotometrically at 595 nm.
The protein-dye complex has a high extinction coefficient, leading to great sensitivity. The protein-dye binding is a very rapid process (approximately 2 minutes) and the complex remains stable in solution for about 1 hour. This method is not usually subject to interference by other components but the presence of large quantities of surfactants (1%) does interfere (Bradford, 1976). This method is highly reproducible (standard deviation 1.2% of mean value).

Preparation of protein reagent

100 mg of Coomassie dye were dissolved in 50 mL of 95% ethanol. 100 mL of 85% phosphoric acid was added and the resulting solution was diluted to a final volume of 1000 mL with distilled water.

Protein assay

To each 0.1 mL protein sample was added 5 mL of protein reagent. After mixing its absorbance was measured at 595 nm against a blank (prepared in the same manner using the appropriate buffer) in a UV-Vis spectrophotometer (Heλios γ Unicam, Cambridge, England). Each assay was done in duplicate or triplicate and the final value is the arithmetic mean of those determinations. BSA was used as the protein for the construction of the calibration curve (Figure 3.4). BSA standards were also included with every set of samples for protein assay.

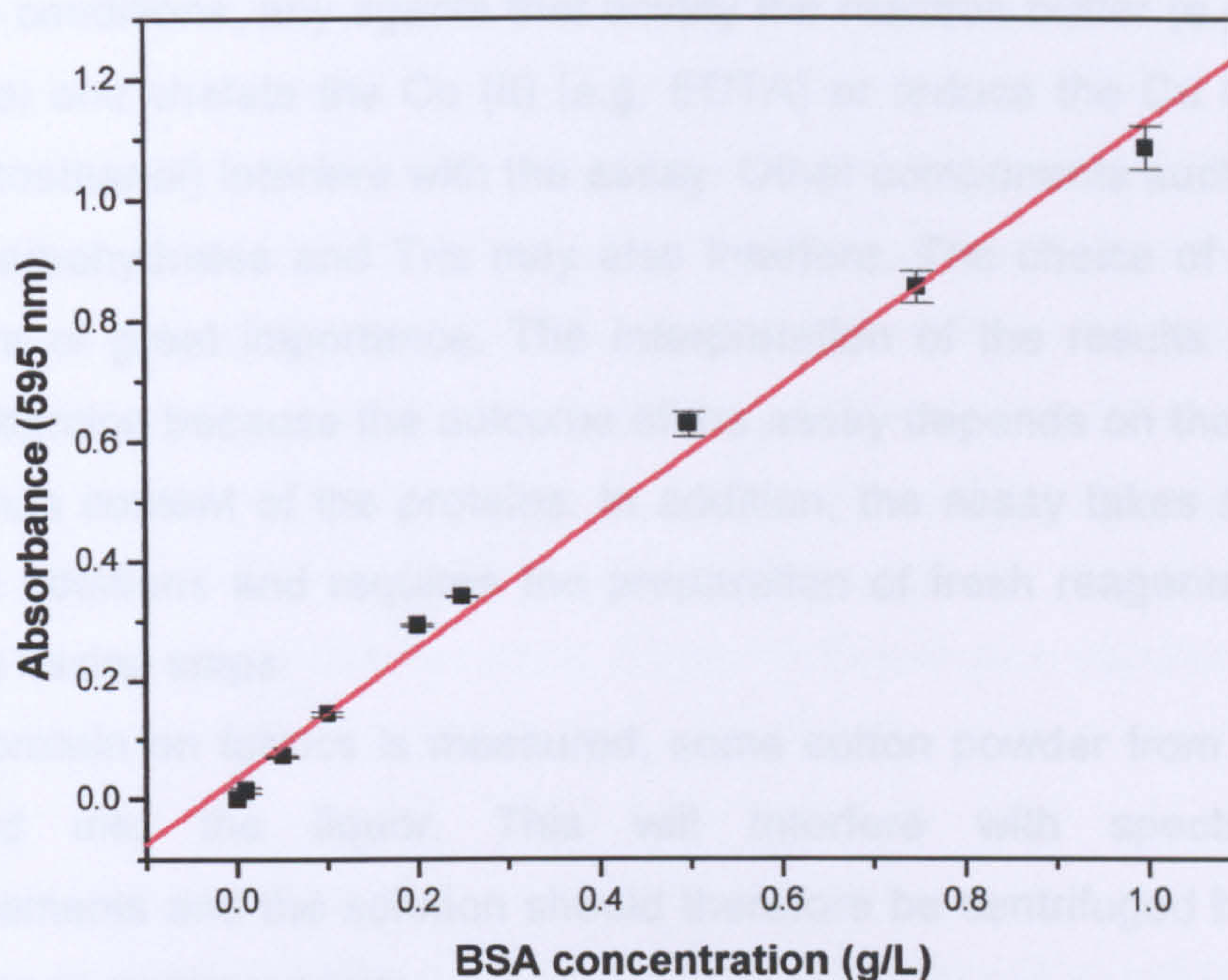


Figure 3.4 Calibration curve for protein determination using the Bradford assay with BSA as the protein standard.

3.2.5 Determination of protein adsorbed to the fabric

Protein adsorbed to the fabric was measured using the Lowry method as modified by Ghose (1987).

Description of the method

This assay relies on two colour reactions: in the first reaction, Cu (II) at alkaline pH forms a purple (Biuret) complex with four nitrogen atoms, two from each of two peptide chains and is reduced to Cu (I). There is little variation from protein to protein but the colour change to purple in not very pronounced. The second reaction leads to the formation of a much more intense blue-green colour, as a

result of the interaction of the phenol reagent with Cu (I) Biuret complexes of tyrosine and tryptophan side chains. Since the mechanism requires Cu in alkaline conditions, any agents that acidify the reaction buffer (e.g. ammonium sulphate) and chelate the Cu (II) (e.g. EDTA) or reduce the Cu (II) (e.g. DTT, mercaptoethanol) interfere with the assay. Other components such as K^+, Mg^{2+}, NH_4^+, carbohydrates and Tris may also interfere. The choice of the blanks is therefore of great importance. The interpretation of the results also requires some attention because the outcome of the assay depends on the tyrosine and tryptophan content of the proteins. In addition, the assay takes some time for reagent additions and requires the preparation of fresh reagents and precise solution mixing steps.

When protein on fabrics is measured, some cotton powder from the fabrics is liberated into the liquor. This will interfere with spectrophotometric measurements and the solution should therefore be centrifuged before making absorbance measurements.

Preparation of reagents

Reagent A: 20 g of sodium carbonate and 4 g of sodium hydroxide were dissolved in distilled water to make a volume of 1000 mL;

Reagent B-1: 1 g of Copper (II) sulphate pentahydrate in 100 mL of distilled water;

Reagent B-2: 2 g of sodium potassium tartrate in 100 mL of distilled water;

Reagent C: 1 mL of reagent B-1, 1 mL reagent B-2 and 100 mL of reagent A. The reagents must be mixed in this order and this reagent should not be kept for more than 24 h.

Phenol reagent (1 *N*): Folin-Ciocalteau phenol reagent (2 *N*) was diluted with an equal volume of distilled water.

Protein assay

0.5 mL of sample was placed in a test tube with 5 mL of reagent C and well mixed. After 10 minutes, 0.5 mL of phenol reagent was added and mixed at once. The absorbance at 750 nm was read (after waiting 30 minutes) against a blank (prepared in the same way) in a UV-Vis spectrophotometer (Heλios γ Unicam, Cambridge, England). When protein in the fabric was being measured, 0.5 ml of sample was substituted by 150 mg of fabric. The determinations were done in triplicate and the final value is the arithmetic mean of those determinations. Bovine serum albumin was used as protein standard for construction of the calibration curve (Figure 3.5) and as a standard for each set of sample assays.

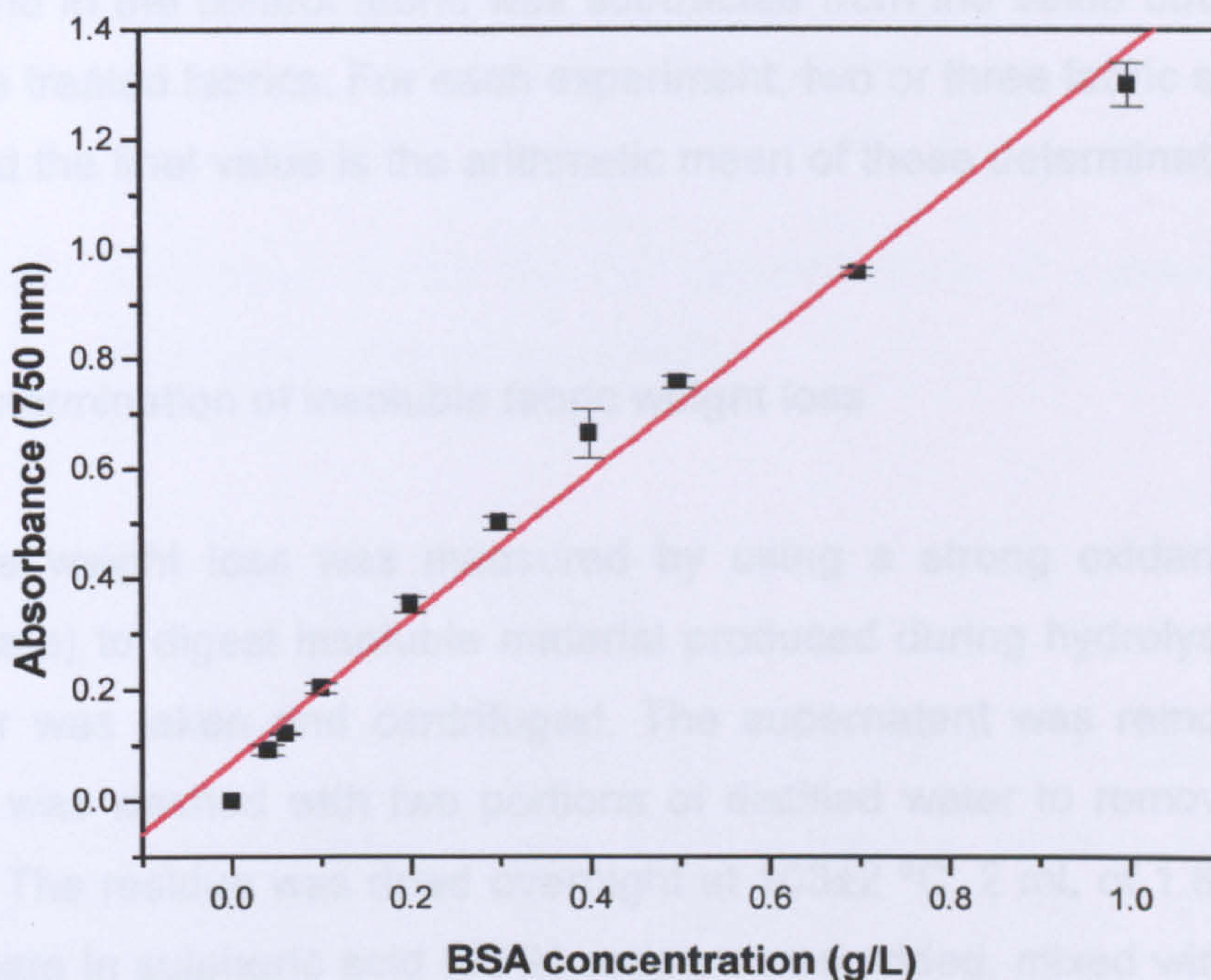


Figure 3.5 Calibration curve for protein determination using the Lowry method with BSA as the protein standard.

3.2.6 Determination of total fabric weight loss

The weight loss was determined by difference in weights of fabric samples before and after treatments and always after conditioning for 24 hours at 20 ºC and 65% of relative humidity. In each experiment a control was included, using a fabric subjected to the same conditions and treatments but without cellulase. Fabric weight loss is calculated in % using the following expression:

$$\text{Weight loss (\%)} = \frac{(W_i - W_f)}{W_i} \times 100$$

Where W_i is the initial fabric weight and W_f is the final weight of the fabric after the enzymatic treatment. When a low level of mechanical agitation (no discs) was used in the experiments the weight loss of the control fabrics was negligible, but when high mechanical agitation was used, the value of weight loss found in the control fabric was subtracted from the value obtained for the cellulase treated fabrics. For each experiment, two or three fabric samples were used and the final value is the arithmetic mean of those determinations.

3.2.7 Determination of insoluble fabric weight loss

Insoluble weight loss was measured by using a strong oxidant (potassium dichromate) to digest insoluble material produced during hydrolysis. A sample of liquor was taken and centrifuged. The supernatant was removed and the residue was washed with two portions of distilled water to remove all soluble sugars. The residue was dried overnight at 103±2 ºC. 2 mL of 1.8% potassium dichromate in sulphuric acid (50%) reagent was added, mixed with the residue and heated on a boiling water bath for 30 minutes. After dilution with 50 mL of distilled water, the absorbance of the solutions was read in an UV-Vis spectrophotometer against a water blank at 441 nm. Glucose was used as a

standard for construction the calibration curve (Figure 3.6). Glucose standards were also included with each set of samples for determination.

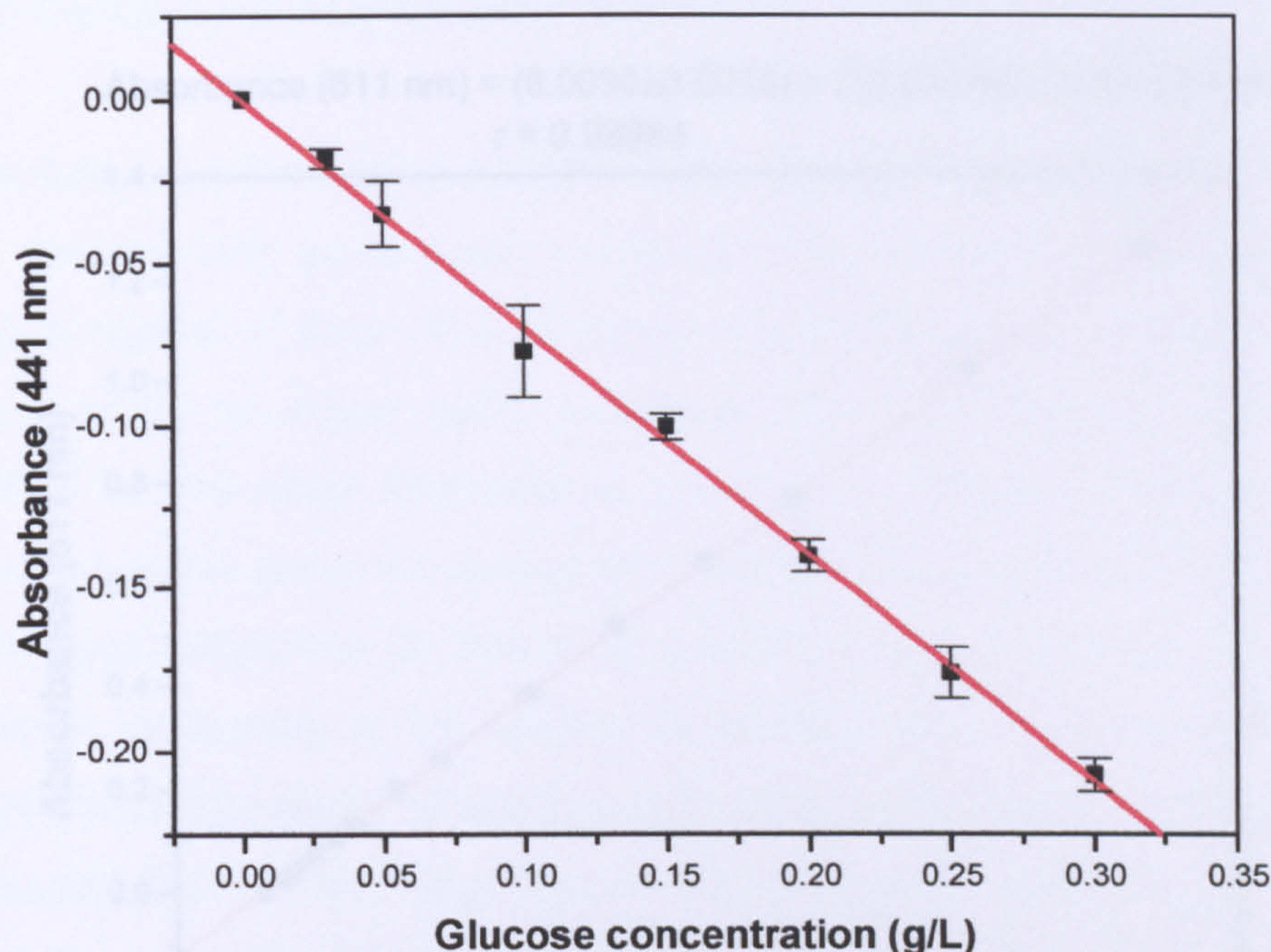


Figure 3.6 Calibration curve for the determination of insoluble cellulosic material measured as glucose.

3.2.8 Determination of dyes in solution

A stock solution (5%) of the dye Remazol Brilliant Blue R was prepared by dissolving the dye in distilled water. From this stock solution, other solutions with different concentrations were prepared by dilution. The maximum wavelength of adsorption of dye was recorded by making a scan between 400 and 800 nm using a UV-Vis spectrophotometer (Heλios α Unicam, Cambridge, England). For this particular dye the wavelength of maximum adsorption obtained was 611 nm. For this wavelength, the absorbance of the different dye

concentration solutions was measured and plotted vs dye concentration (see Figure 3.7). The concentration of dye in the samples was then measured by reading the absorbance at 611 nm and calculated from the calibration curve.

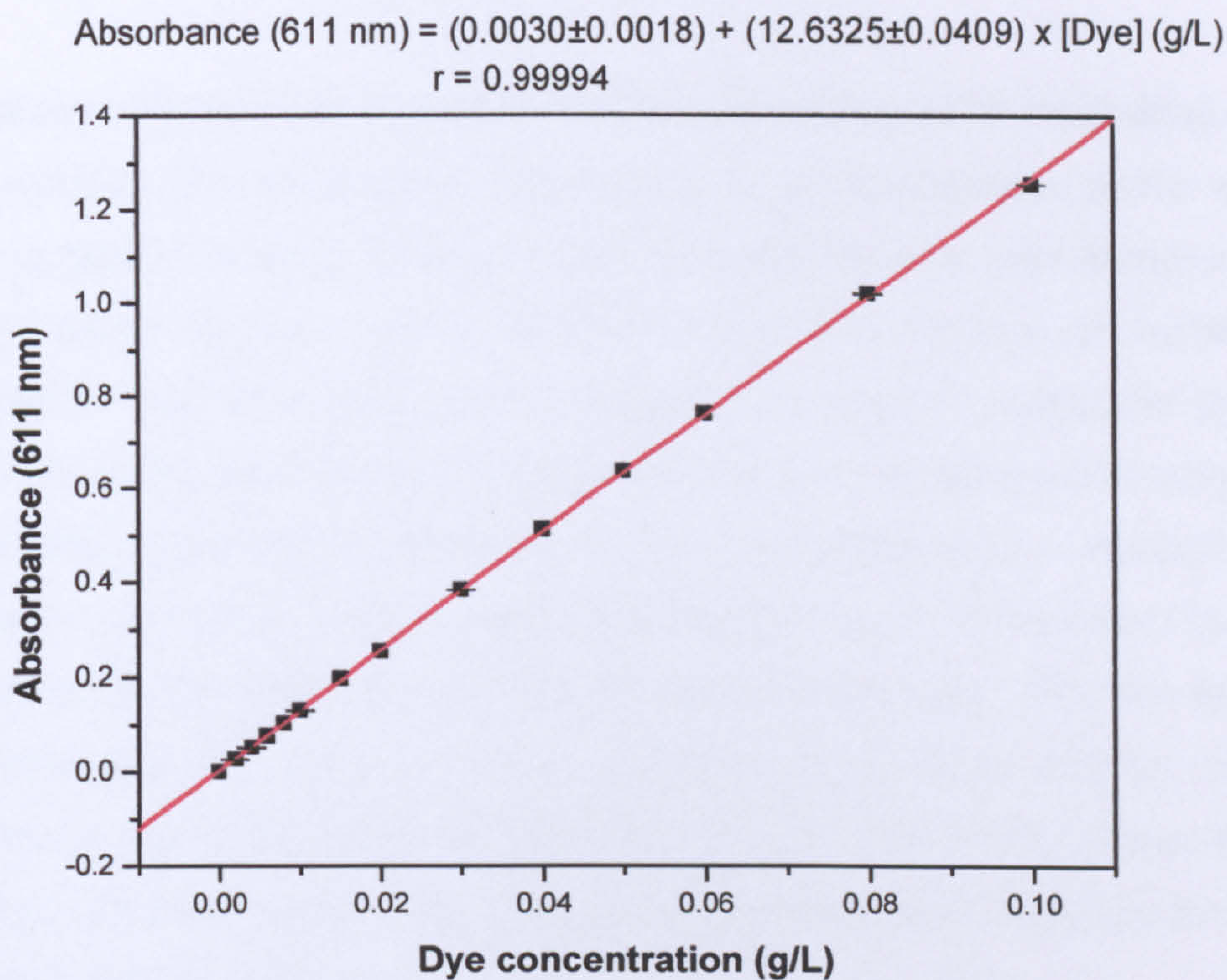


Figure 3.7 Calibration curve for dye Remazol Brilliant Blue R.

3.2.9 Determination of cellulase adsorption

Samples of the initial reaction mixture (cellulase in buffer solution) and after enzymatic treatments were taken to measure total protein in solution (Bradford assay) and hence protein adsorption by difference. Percentage of adsorption was calculated by using the expression:

$$\text{Enzyme adsorption (\%)} = \frac{(E_i - E_f)}{E_i} \times 100$$

where E_i is the initial protein concentration in solution and E_f is the protein concentration after the enzymatic treatment.

3.2.10 Cellulase activity on cotton fabrics

The activity of cellulases on cotton fabrics was measured by incubating a cotton fabric sample with an enzyme preparation in an appropriate buffer at 50 °C during a period of time. The cellulase preparation and concentration, buffer system, fabric to liquor ratio, treatment time and degree of agitation are specified for individual experiments (chapter 4). Control treatments were also carried out in the same incubation conditions but in the absence of enzymes.
Activity was measured in terms of soluble reducing sugars released during hydrolysis, according to the method described in 3.2.1. Sometimes the activity was expressed in terms of fabric weight loss. In this case, after the enzymatic treatments, the residual enzymes on the fabric were inactivated by immersing the fabric in a sodium carbonate solution (5%, w/v) and further rinsed in boiling and cold distilled water. The fabrics were dried and weighed for activity evaluation (see 3.2.6).
The activity of cellulases on cotton fabrics was also sometimes evaluated by determination of the cotton reducing power (3.2.2).
When significant amounts of insoluble fragments (insoluble weight loss) were produced during the enzymatic treatments of cotton fabrics, the insoluble residue was evaluated by the method described in 3.2.7.

3.2.11 Activity on carboxylmethylcellulose (endoglucanase activity)

The endoglucanase activity of cellulase samples was measured towards CMC according to the following procedure: 1 mL of cellulase sample was placed in a test tube and pre-incubated in a water bath at 50 °C. The same amount of CMC solution (0.5%, dissolved in the appropriate buffer) was added to the test tube

and the incubation was carried out at 50 °C for 30 minutes at 125 rpm (Shaker bath OLS 200, Grant Instruments, Cambridge, UK). The reducing sugars released were then determined by the method described in 3.2.1 and the activity was expressed in terms of g/L of reducing sugars produced.

3.2.12 Activity on filter paper (total cellulase activity)

Activity on FP was used to measure the activity of total cellulase systems. Cellulase samples (1 mL) were pre-heated at 50 °C in appropriate buffers. The reaction was started by adding filter paper discs (made with an office perforator, 5 mm diameter, 3.0±0.5 mg) and the incubation was made at 125 rpm in a shaker bath at 50 °C. After 1 hour, the reaction mixture was analysed for reducing sugars using the method described in 3.2.1 and the activity was expressed in terms of g/L of reducing sugars produced. Controls containing buffer (2 mL) and filter paper only were treated similarly.

3.12.13 Activity on cellobiose (cellobiase activity)

The β-glucosidase activity was measured using cellobiose as substrate. The incubation was performed by placing cellulase preparations in 100 mL of cellobiose solution (prepared in acetate buffer, 0.1 *M*, pH 5.0). The mixture was incubated in a water bath shaker at 50 °C and operating at 125 rpm. Samples were collected and the enzymatic reaction was stopped by putting the sample flasks in a boiling water bath for 5 minutes. The resulting glucose concentration was measured by the glucose oxidase method using the method described in 3.2.3. Activity was expressed in terms of enzyme units (U), micromoles of glucose produced per minute.

3.2.14 Dyeing procedures

Dyeing with Reactive Blue 19 was performed at 4% dye o.w.f at 10:1 liquor ratio in presence of sodium chloride (70 g/L) and sodium carbonate (5 g/L) at 60 °C for 60 minutes. After 20 minutes 2 g/L sodium hydroxide (50%) was added to the dye bath. Dyed fabrics were washed with a nonionic surfactant 0.5 g/L (Lutensol ON 30, BASF, Ludwigshafenrhein, Germany) at 100 °C for 30 minutes to remove unfixed dye.

Dyeing with Vat Blue 1 was performed at 16:1 liquor ratio in presence of sodium dithionite and sodium hydroxide. The preparation of the vat solution was performed by incubating the dyeing bath at 55 °C for 15-20 minutes. After the formation of indigo leuco form (yellow solution) the cotton fabric was added to the dye bath and dyeing was carried out for another 20 minutes in the same conditions. After this period, the fabric was air oxidized and then incubated again in a new vat solution to obtain a deeper shade. After air oxidation, the dyed fabrics were washed at 100 °C in presence of a nonionic surfactant 0.5 g/L (Lutensol ON 30, BASF, Ludwigshafenrhein, Germany) to remove surface dye and to aid migration and aggregation of the oxidised indigo in the fibre.

4. INDIVIDUAL EXPERIMENTS WITH RESULTS AND DISCUSSION

4.1 Preliminary recycling experiments

After an enzymatic treatment of cotton with cellulases, the enzymes are distributed between the liquor and the substrate (cotton cellulose). This means that cellulases may be recoverable from either phase.
This part of the work describes some preliminary recycling experiments which were carried out in order to determine the relative amounts of recyclable enzyme present in the liquor and adsorbed to the substrate, and to assess the loss in cellulase activity occurring after several recycling steps. Two types of recycling strategies were investigated: recycling cellulases in the liquor and recycling cellulases adsorbed on the fabric.

Enzymatic hydrolysis

Fabric samples ($\approx$ 7 g) were placed in stainless pots (500 mL) of a Rotawash machine with 0.6 mL of stock enzyme solution (TC from *Trichoderma reesei*) in 100 mL of acetate buffer (0,1 *M*, pH 5.0). The pots were rotated at 40 rpm during 1 hour. After this period, the fabric was removed from the liquor and treated as described in 3.2.10 for determination of weight loss. The liquor, before and after treatment, was analysed for soluble sugars (3.2.1) and protein (3.2.4) content. All experiments were done in duplicate.

Recycling cellulases in the liquor

In this strategy the fabric was removed after 1 hour of treatment and a new fabric was added to the liquor. Cellulase recycling was carried out four times (*i.e.* a total of five hydrolyses) as shown in Figure 4.1. Each time that a fabric was removed, the free liquor was allowed to drain off into the reaction vessel, but no attempt was made to squeeze out all of the liquor. The volume of the

liquor was not readjusted after removal of fabrics and consequently the liquor volume was reduced by about 50% over the five hydrolysis cycles. Fabric weight loss and total protein and soluble sugars in the liquor were determined at the end of each step.

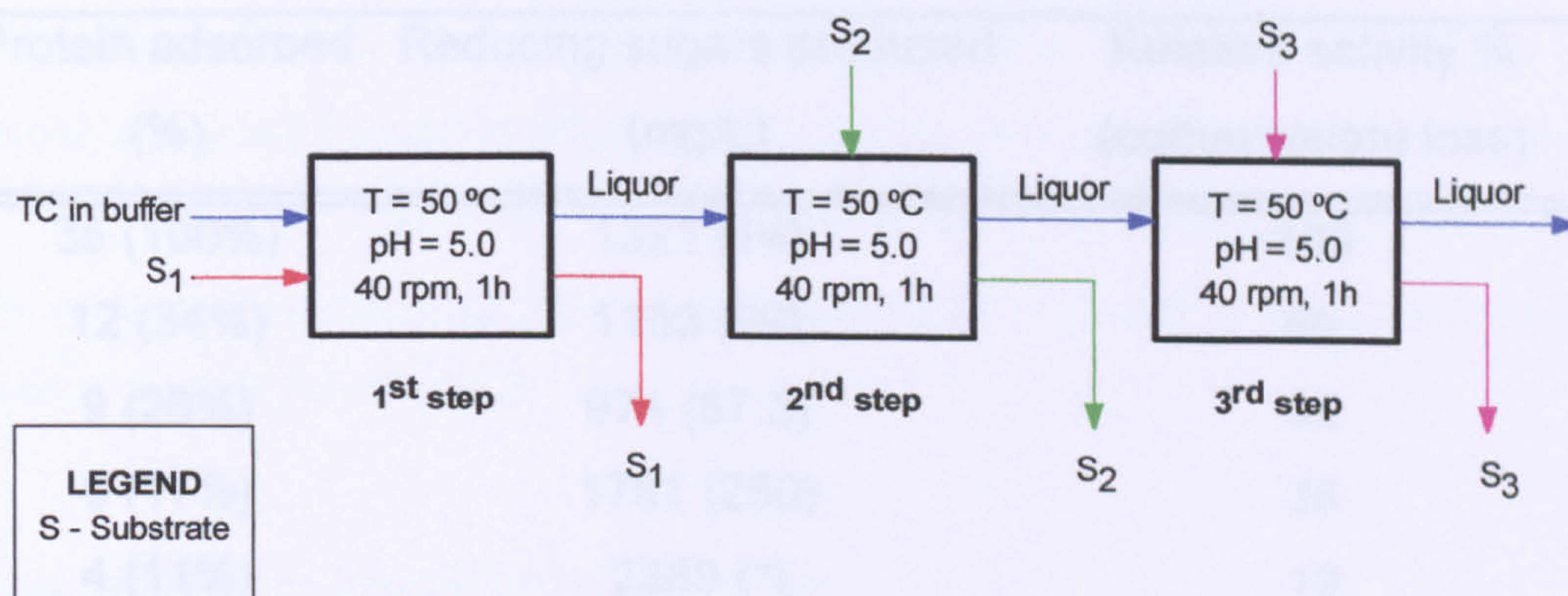


Figure 4.1 Schematic diagram for the recycling of cellulases in the liquor.

Table 4.1 Recycling of cellulases in the treatment liquor (TC from *T. reesei*). Concentration of protein and soluble reducing sugars in the liquor at the end of each recycling step (50 ºC, pH 5.0, 1 h) and the weight loss of each fabric sample. Values in brackets represent the standard deviation of two independent experiments.

Step	[Protein] (mg/L)	[Reducing sugars] (mg/L)	Weight loss (%)
0	194.6 (2.6)	320.0 (12.1)	-
1	125.7 (0.4)	1641.6 (26.5)	S_1: 2.6 (0.1)
2	110.8 (5.3)	2795.2 (72.4)	S_2: 1.8 (0.2)
3	101.7 (1.5)	3769.2 (15.1)	S_3: 1.2 (0.2)
4	96.1 (1.5)	5550.3 (235.3)	S_4: 1.0 (0.1)
5	92.6 (*)	8105.8 (*)	S_5 : 0.5 (0.1)

* result of a single experiment.

Table 4.2 Recycling of cellulases in the treatment liquor (TC from *T. reesei*). Percentage of protein adsorbed, soluble reducing sugars produced and relative activity in terms of fabric weight loss in each recycling step.

Step	Protein adsorbed (%)	Reducing sugars produced (mg/L)	Relative activity % (cotton weight loss)
1	35 (100%)	1321 (14)	100
2	12 (34%)	1153 (99)	69
3	9 (26%)	974 (57.3)	46
4	6 (17%)	1781 (250)	38
5	4 (11%)	2389 (*)	19

* result of a single experiment.

It was observed, after the first treatment, that about 65% of the protein remained in the liquor and hence, by difference, 35% was adsorbed to the substrate, which was discarded in this case. In subsequent recycling steps much greater (and increasing) amounts of cellulase were retained in the liquor (Tables 4.1 and 4.2) indicating decreasing adsorption to the substrate with successive recycling steps. This seems to be consistent with the declining cellulase activity towards the cotton substrates as measured by weight loss, but not with the rate of increase of reducing sugars. Allowing for the reduction in volume of the treatment liquor, the activity measured as reducing sugars appeared to be significantly reduced by the third hydrolysis, but to increase to its original level by the fifth hydrolysis. Reduced adsorption and cotton weight loss may be explained by reaction products (soluble oligosaccharides, cellobiose and glucose) remaining bound to the EGs and CBHs and inhibiting their adsorption to the fabric. Such effects have been noted by Lee and Fan (1983). Further experiments to examine the effects of end products are described in 4.2. The relatively constant rate of release of reducing sugars may be explained by conversion of cellobiose to glucose. A study of the kinetics of

cellobiose conversion to glucose by β-glucosidase present in the TC preparation is also reported in 4.2.

Alternatively, the progressive reduction in activity towards cotton substrates, may be explained by a loss of synergy between EGs and CBHs. This could occur if one component becomes depleted by preferential and or irreversible adsorption. Experiments to investigate this possibility are described in 4.4. Mechanical, shearing forces or thermal effects could also account for cellulase activity loss, but this would not be consistent with the continual release of reducing sugars. Thermal and mechanical deactivation under textile processing conditions is investigated in 4.3.

Recycling cellulases adsorbed to the fabric

This strategy is represented in Figure 4.2. After 1 hour of hydrolysis (as described above), the treated fabric (S_1) was removed from the liquor and placed in a fresh buffer solution (without enzyme) together with a new fabric sample (S_2). A second round of treatment was then initiated. After hydrolysis, S_1 was removed for weight loss determination and S_2 was transferred to fresh buffer, with a further new fabric sample (S_3). A third hydrolysis step was carried out. Fabrics S_2 and S_3 were than analysed for weight loss. Concentration of cellulase protein and liberated reducing sugars were determined after each hydrolysis step.

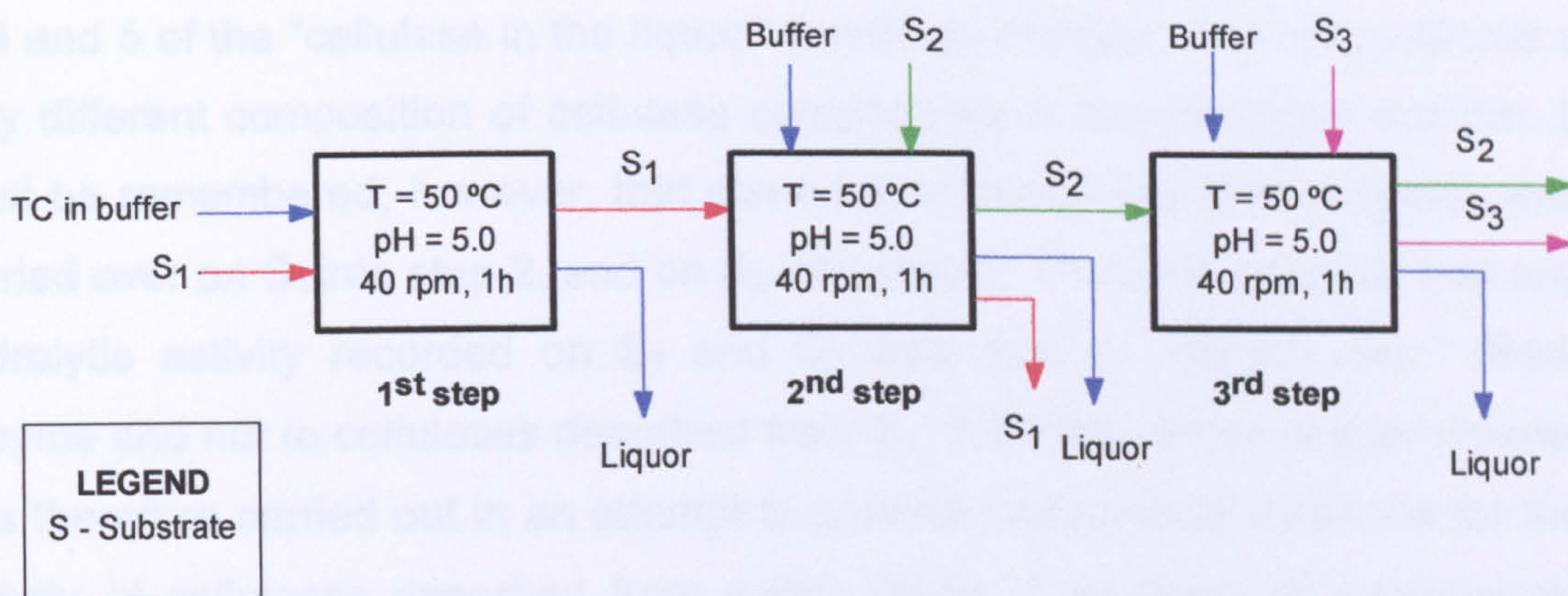


Figure 4.2 Schematic diagram for the recycling of cellulases adsorbed on the fabric.

Table 4.3 Recycling of cellulases adsorbed on the fabric (TC from *T. reesei*). Concentration of protein and soluble reducing sugars in the liquor at the end of each recycling step (50 ºC, pH 5.0, 1 h) and the weight loss of each substrate. Values in brackets represent the standard deviation of two independent experiments.

Step	[Protein] (mg/L)	[Reducing sugars] (mg/L)	Weight loss (%)
0	202.4 (2.7)	213.3 (11.2)	-
1	132.8 (6.5)	1217.2 (20.0)	S_1: 2.5 (0.0)
2	14.5 (1.7)	602.7 (65.9)	S_2: 1.0 (0.1)
3	0.14 (0.2)	251.6 (20.7)	S_3: 0.2 (0.0)

The aim of this experiment was only to determine whether cellulase adsorbed on cotton fabric retains its activity and can be desorbed and recycled. The difference in liquor ratio between step 1 and step 2 and 3 was therefore considered not to have any significance for interpretation of the results, which at first glance (Table 4.3) appear to confirm the activity on S_2 of cellulase desorbed from S_1. It is also worth noting that the percentage of protein adsorbed in step 3 (and the absolute amount) was much greater than in steps

3, 4 and 5 of the "cellulase in the liquor" recycling strategy. This may indicate a very different composition of cellulase components in the desorbed fraction. It must be remembered, however, that some liquor containing free enzymes was carried over on S_1 into step 2, and on S_2 into step 3. Thus it is possible that any hydrolytic activity recorded on S_2 and S_3 was due to "carried over" (free) enzyme and not to cellulases desorbed from S_1. A further series of experiments was therefore carried out in an attempt to provide unequivocal evidence for the activity of cellulases desorbed from cotton fabric. Two types of experiments were performed and they are represented in Figures 4.3 and 4.4.

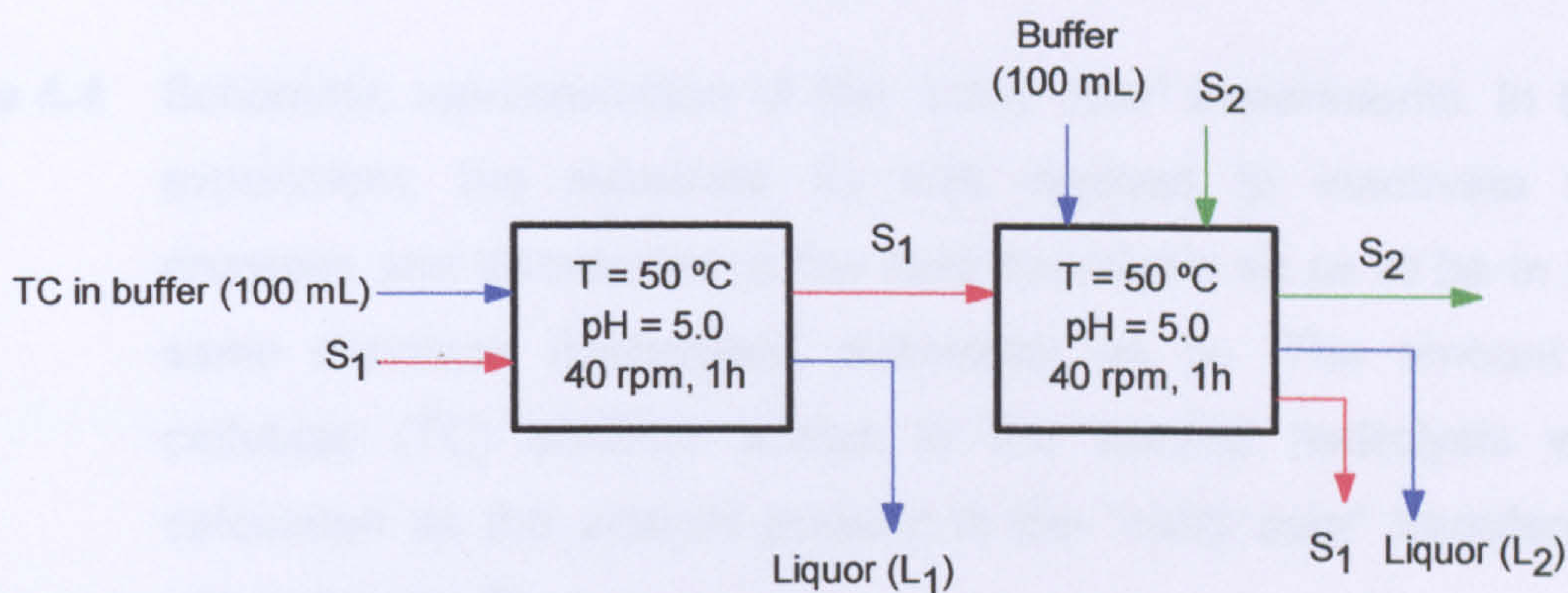


Figure 4.3 Schematic representation of the "carry over" experiments. In this experiment, the substrate S_1 with adsorbed enzymes plus carried over enzymes (in buffer solution) were transferred to the next hydrolysis.

Comparing the values of weight loss achieved for the substrates S_2 and S_4 (Table 4.4), it can be seen that the value of S_2 is slightly greater, which means that the enzymes adsorbed to the substrate S_1 probably contribute to the weight loss found for S_2. This is confirmed by the analysis of the reducing sugars in liquor L_4 (Table 4.5) produced in the second hydrolysis represented in Figure 4.4. Since this value is considerably lower than that found in liquor L_2 (Table 4.5) obtained in the second hydrolysis of the experiment describe in Figure 4.3 it is clear that cellulases adsorbed on substrate S_1 are active and contribute to

the second hydrolysis step even though they may largely remain adsorbed on substrate S_1.

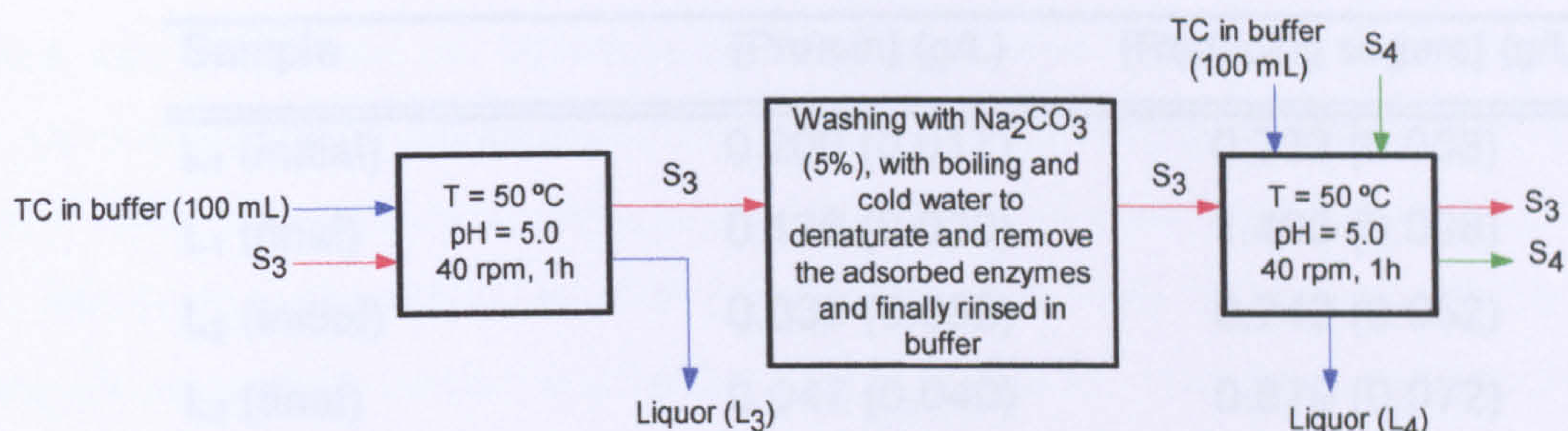


Figure 4.4 Schematic representation of the "carry over" experiments. In this experiment, the substrate S_3 was washed to inactivate the enzymes and transferred to the next hydrolysis so as to be in the same condition (hydrolysed substrate) as S_1. The amount of cellulase (TC) solution added in the second hydrolysis was calculated as the amount present in the "carry over" transferred with substrate S_1.

Table 4.4 Weight loss of the various fabric samples used in the "carry over" experiments. Values in brackets represent the standard deviation of thee independent experiments.

Fabric sample	Weight loss (%)
S_1	3.66 (0.44)
S_2	1.29 (0.07)
S_3	3.80 (0.33)
S_4	1.11 (0.08)

Table 4.5 Protein and reducing sugars in solution for the different samples in the carry over experiments.

Sample	[Protein] (g/L)	[Reducing sugars] (g/L)
L_1 (initial)	0.200 (0.017)	0.232 (0.003)
L_1 (final)	0.126 (0.020)	1.493 (0.098)
L_2 (initial)	0.039 (0.033)	0.242 (0.052)
L_2 (final)	0.047 (0.040)	0.879 (0.072)
L_3 (initial)	0.201 (0.019)	0.230 (0.008)
L_3 (final)	0.135 (0.021)	1.498 (0.062)
L_4 (initial)	0.043 (0.032)	0.063 (0.014)
L_4 (final)	0.042 (0.036)	0.525 (0.030)

4.2 Cellulase inhibition by end products

4.2.1 End-product inhibition studies

When cellulases remaining in the liquor used for treating cotton fabrics were recycled (Figure 4.1) there was a progressive reduction in adsorption of cellulase to fabric, and in fabric weight loss, throughout the four recycling steps (Table 4.1). It was suggested that this could be caused by "end-product inhibition" effects which are widely reported in the literature (2.3.2.1).
The aim of this study was to investigate the extent of inhibition, of the cellulase system produced by the fungus *T. reesei*, by glucose and cellobiose at inhibitory sugar concentrations which might be expected to occur after cotton processing and recycling of the liquor.

Prior to use, glucose and cellobiose were dried in an oven (103 ± 2 °C, 4 hours).

Enzymatic treatments of cotton fabrics were performed by incubating ≈ 7 g of fabric with TC (3.8 mg protein/g fabric) in 100 mL of acetate buffer (0.1 *M*, pH 5.0) in a Rotawash machine (50 °C, 20 rpm, 1 h). To study the influence of glucose and cellobiose on cellulase activity, the same incubation procedures were carried out but in presence of increasing concentrations of the sugars (0-30 g/L). After the enzymatic treatments, the residual enzymes on the fabric were inactivated according to the procedure described in 3.2.10. Control treatments were also carried out in the same incubation conditions but in the absence of enzymes (in buffer or sugar solution only).

Samples of the reaction mixtures, before and after enzymatic treatments, were taken to measure total protein in solution and then calculate protein adsorption by difference (see 3.2.9).

Cellulase activity towards cotton fabrics was measured in terms of fabric weight loss (Table I in Appendix A) and was expressed, in terms of percentage, relative to the activity obtained in the absence of sugars.

The results are represented in Figures 4.5 and 4.6 from which it is possible to observe that both sugars inhibited the activity of cellulases. Cellobiose shows a stronger inhibition effect than glucose and its inhibitory effect was significant even at low concentrations. With 5 g/L of cellobiose present in the initial hydrolysis reaction, there was a 25% reduction in cellulase activity. With 20 g/L of cellobiose, the cellulase activity was reduced to half the value obtained without any sugars present at the beginning of reaction. The effect of glucose was less severe and even in the presence of 30 g/L of glucose the total cellulase still retained 78% of its initial activity.

Other workers have found comparable results; Holtzapple *et al.* (1990), for example, found that cellulases from *T. reesei* retained 37% of their activity in a 55% glucose solution and that cellobiose caused more inhibition than glucose.

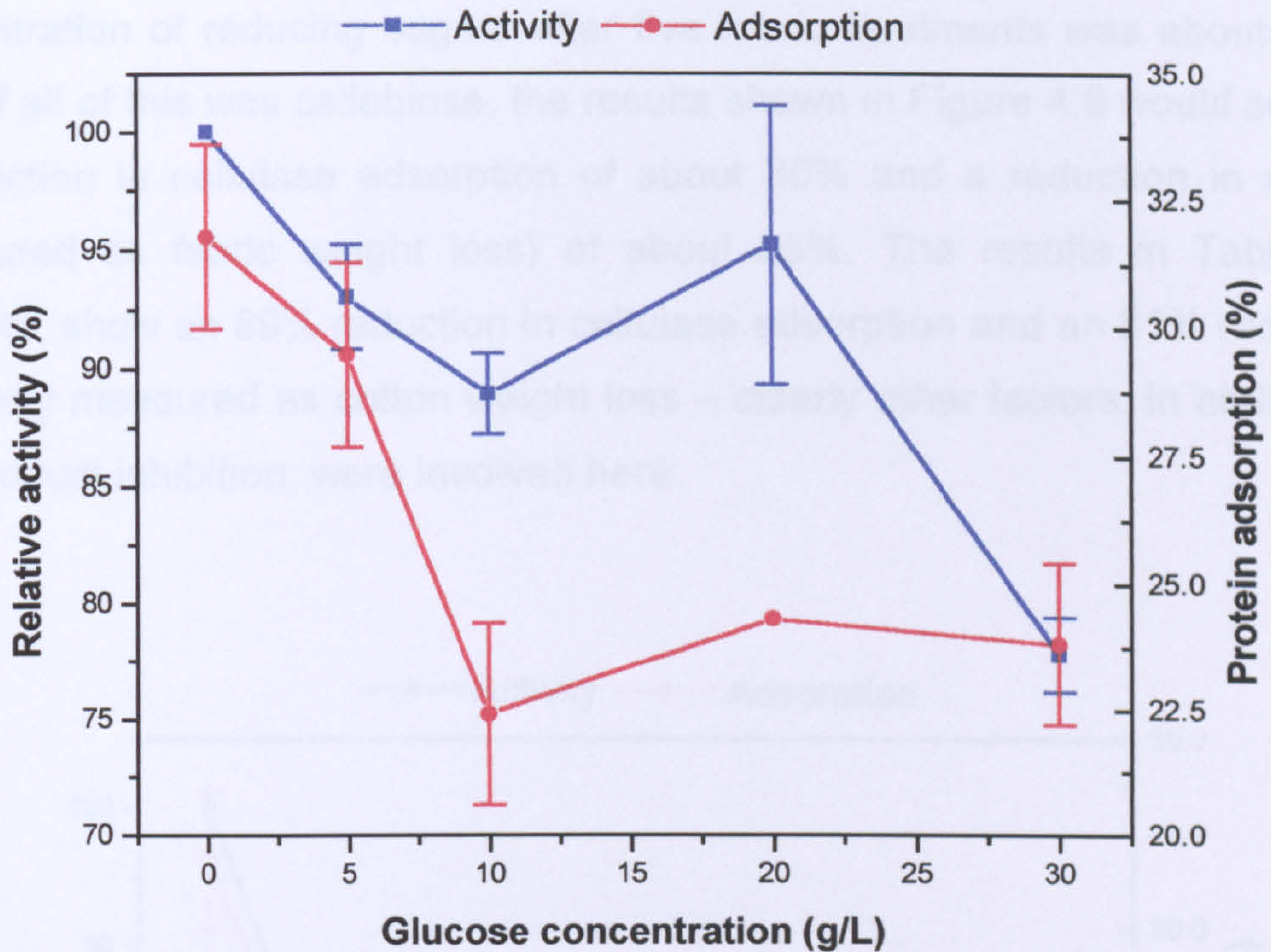


Figure 4.5 Effect of glucose concentration on cellulase (TC) activity (as fabric weight loss) and adsorption (50 ºC, pH 5.0, 3.8 mg protein/g fabric, 1 h). The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

Vallander and Ericksson (1987) reported that cellulases are not inhibited by end products until the sugar concentration reaches about 6%, but the sugar inhibition concentration depends on the proportion of glucose and cellobiose in the total sugars. In this work the authors supplemented the cellulolytic system with an *Aspergillus niger* β-glucosidase, so that the cellobiose produced during hydrolysis was more effectively converted to glucose.

Lee and Fan (1983) studied the effect of glucose and cellobiose on the rate of cellulose hydrolysis by *Trichoderma reesei* cellulases and found that the apparent hydrolysis rate dropped from 9.1 $gL^{-1}h^{-1}$ to 1.4 $gL^{-1}h^{-1}$ when 30 g/L of cellobiose were added, and to 3.0 $gL^{-1}h^{-1}$ when 30 g/L of glucose were added. They also found that the presence of the sugars slightly reduced the extent of protein

adsorption. In the preliminary recycling experiments (Table 4.1) the total concentration of reducing sugars after five fabric treatments was about 8 g/L. Even if all of this was cellobiose, the results shown in Figure 4.6 would suggest a reduction in cellulase adsorption of about 30% and a reduction in activity (measured as fabric weight loss) of about 35%. The results in Table 4.2, however, show an 89% reduction in cellulase adsorption and an 81% reduction in activity measured as cotton weight loss – clearly other factors, in addition to end product inhibition, were involved here.

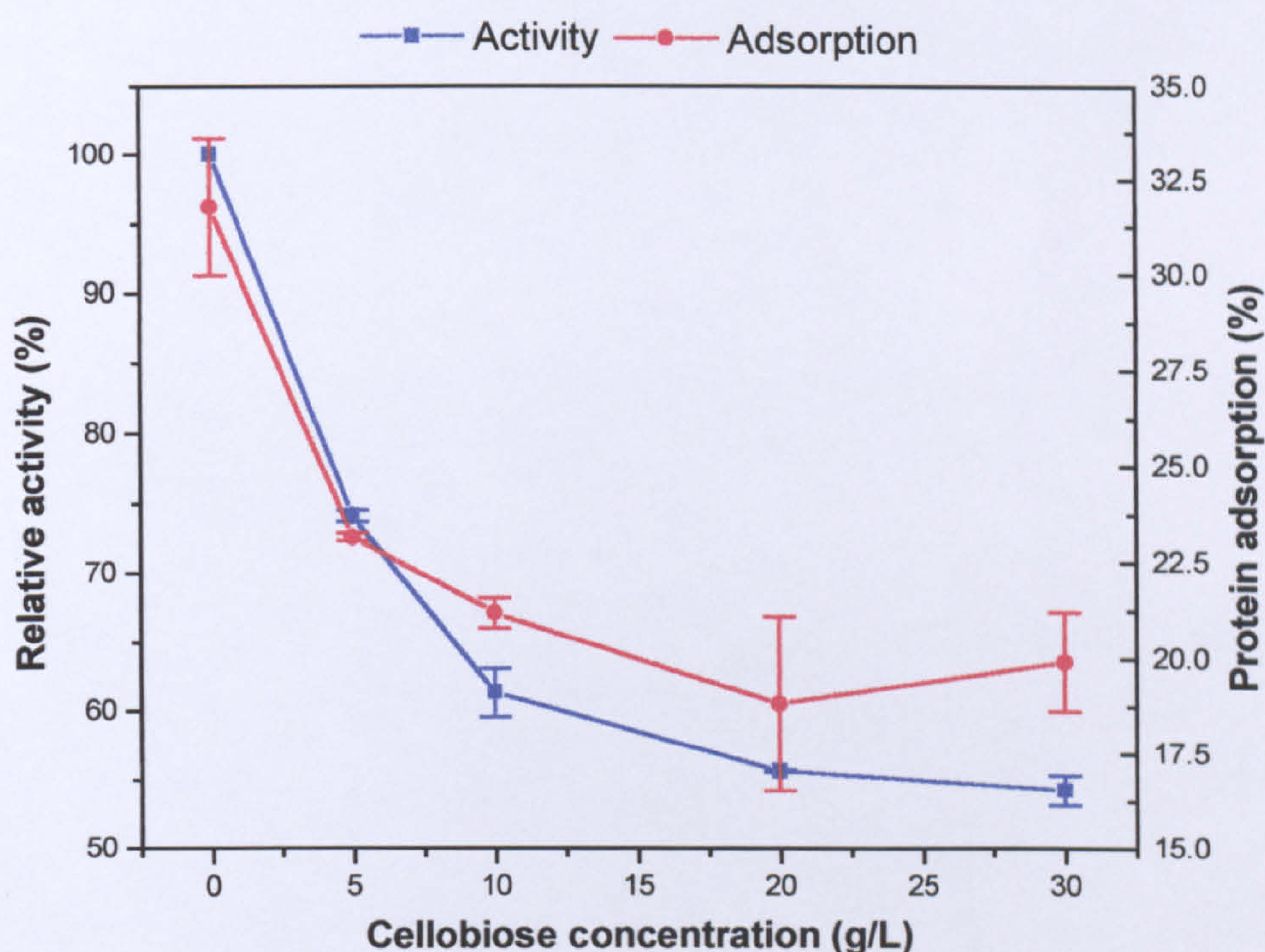


Figure 4.6 Effect of cellobiose concentration on cellulase (TC) activity (as fabric weight loss) and adsorption (50 ºC, pH 5.0, 3.8 mg protein/g fabric, 1 h). The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

The results shown in Tables 4.1 and 4.2, for the production of reducing sugars, must be interpretated with caution, because the volume of the reaction solution

was decreasing with each recycling step. Nevertheless there are indications that by the third fabric treatment there was a marked reduction in the rate of production of reducing sugars which then increased again in the fourth and fifth treatments. This could possibly be accounted for by the increasing fraction of cellulase remaining in solution contributing more to the hydrolysis of soluble oligomers.

If efficient recycling is to be achieved, it is important to understand more about these effects. It was therefore decided to determine the the relative amounts of glucose and total reducing sugars produced by TC, EG-rich and CBH-rich cellulases during cotton hydrolysis (see 4.2.2) and the rate of conversion of cellobiose to glucose, by the β-glucosidase present in TC (see 4.2.3) before going on to study other possible reasons for the greater than expected losses in activity on recycling (see 4.3 and 4.4).

4.2.2 Total reducing sugars and glucose produced by TC, EG-rich and CBH-rich cellulases ex *T. reesei*

The activities on cotton fabrics of TC, EG-rich and CBH-rich cellulases from *T. reesei* were characterized in terms of fabric weight loss, liberation of total reducing sugars, and liberation of glucose. Fabric samples (≈ 6.6 g) were incubated in the cellulase mixtures diluted in acetate buffer (0.1 *M*, pH 5.0) in a Rotawash machine, at 50 °C during 1 hour. All the treatments were done in duplicate. After incubation, reducing sugars were determined by the neocuproine method (3.2.1) and glucose by the glucose oxidase method (3.2.3). The percentage of protein adsorption was determined as described in 3.2.9 and fabric weight loss was determined as described in 3.2.10 and 3.2.6.

The results given in Table 4.6 show that after a typical enzymatic treatment of cotton using a TC cellulase preparation, glucose represents 55% of total reducing sugars measured in the reaction mixture (Table 4.6). These ratios

change with the cellulase preparation used; with EG-rich, glucose accounted for 36% of the sugars whereas with CBH-rich glucose accounted for 66% of the liberated sugars. These results suggest that after a single fabric treatment the concentration of sugars is not sufficient to cause inhibition and it should be possible to recycle the liquor several times (without removing the end products) with little loss of activity. This, however, is not consistent with the results of the preliminary recycling experiments shown in Table 4.1 and it seems likely that other factors such as thermal and mechanical ageing or loss of synergism between EGs and CBHs may contribute to the reduced activity towards cotton fabric.

Table 4.6 Characterization of TC, EG-rich and CBH-rich cellulases in terms of fabric weight loss, protein adsorption, reducing sugars and glucose production (Rotawash machine, 50 °C, 40 rpm, 1 h). Values in brackets represent the standard deviation of two independent experiments.

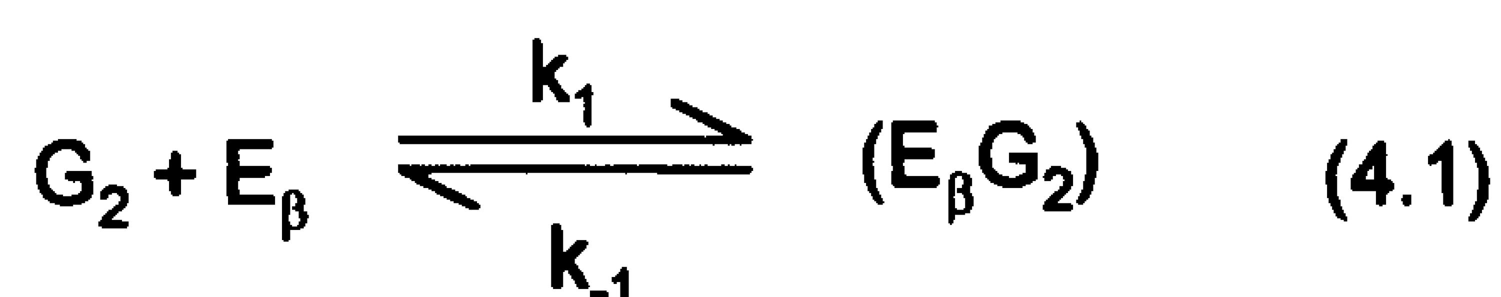
Cellulase preparation	Enzyme dosage (mg protein/ g fabric)	Weight loss (%)	Protein adsorption (%)	[Reducing sugars] (g/L)	[Glucose] (g/L)
TC	2.66 (0.03)	2.96 (0.18)	25.1 (1.0)	1.50 (0.03)	0.83 (0.08)
EG-rich	3.43 (0.12)	1.51 (0.60)	19.0 (3.9)	0.83 (0.06)	0.31 (0.05)
CBH-rich	2.46 (0.05)	1.78 (0.13)	33.1 (1.1)	0.99 (0.04)	0.66 (0.04)

The protein adsorption results (Table 4.6) indicate a greater adsorption of CBHs than EGs and if this is also the case in TC then the cellulase composition and hence the synergy between components may change with each recycling step. Further experiments in this area are described in 4.4.

4.2.3 Kinetics of cellobiose hydrolysis by β-glucosidase

Since β-glucosidase is responsible for the degradation of cellobiose to glucose and the former is known to be a strong cellulase inhibitor, it is important to examine the activity of the cellobiase present in crude mixtures. It is known that cellulase preparations of *T. reesei* are relatively deficient in β-glucosidase activity. Assay of β-glucosidase kinetics can provide information on the need for addition of exogenous β-glucosidase activity in order to decrease the inhibition effects of cellobiose. It has been reported that β-glucosidase is also inhibited by glucose (Calsavara *et al.*, 1999; Dekker, 1986).

The kinetics of β-glucosidase activity were studied by placing 0.6 ml of a TC cellulase preparation in 100 mL of cellobiose solution (prepared in acetate buffer, 0.1 *M*, pH 5.0). Cellobiose concentrations in a range between 0.25 and 2 g/L were used. The mixture was incubated in a water bath shaker at 50 °C operating at 125 rpm. Samples were collected in flasks at different periods of time. The enzymatic reaction was stopped by putting the sample flasks in a boiling water bath for 5 minutes. The resulting glucose concentrations were measured by the glucose oxidase method (3.2.3). Since cellobiose is a soluble substrate, kinetic expressions for homogeneous enzymatic reactions can be applied to the hydrolysis of cellobiose. The mechanism of cellobiose decomposition can be written in the following way:



$$G_2 + E_\beta \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E_\beta G_2) \qquad (4.1)$$

$$(E_\beta G_2) \xrightarrow{k_2} E_\beta + 2G_1 \qquad (4.2)$$

Where E_β is the free β-glucosidase enzyme, G_2 is cellobiose (substrate) and G_1 is glucose (product). $E_\beta G_2$ is the enzyme-substrate complex.

In this model inhibition by substrate and or product is not considered. In the literature (Calsavara *et al.*, 1999; Dekker, 1986) it is reported that substrate inhibition occurs for concentrations of cellobiose greater than 10 m*M* (3.42 g/L). In this study a maximum concentration of 2.0 g/L of cellobiose was used.
The mechanism described in equations (4.1) and (4.2) leads to the well known Michaelis-Menten equation:

$$v = \frac{v_{max}[S]}{K_m + [S]} \qquad (4.3)$$

where $K_m = (k_{-1}+k_2)/k_1$ and is called the Michaelis constant. [S] is the substrate concentration, which in this case is cellobiose (G_2).
By inverting equation (4.3) the Lineweaver-Burk expression is obtained:

$$\frac{1}{v} = \left(\frac{K_m}{v_{max}}\right)\frac{1}{[S]} + \frac{1}{v_{max}} \qquad (4.4)$$

By plotting 1/v vs 1/[S] it is possible to obtain a linear regression where K_m/v_{max} represents the slope and the intercept is $1/v_{max}$. v_{max} is the maximum rate and the parameter K_m is the substrate concentration at which the rate is half of its maximum. K_m is described as the affinity of the enzyme for the substrate.

The initial rate (v_0) is usually calculated from tangents drawn through the origin to the curves of product formation versus time. For simplicity, the initial rates were determined on the basis of a single measurement of the amount of product produced, rather than by the tangent method. This approach is valid over only the short period of time when the reaction is proceeding effectively at constant rate. This linear section comprises, at the most, the first 10% of the total possible change and clearly the error is smaller the sooner the rate is measured (Wilson and Walker, 1994). In this case the first 10 minutes (Figure 4.7) was used for calculation of the rate of glucose formation (Figure 4.8).

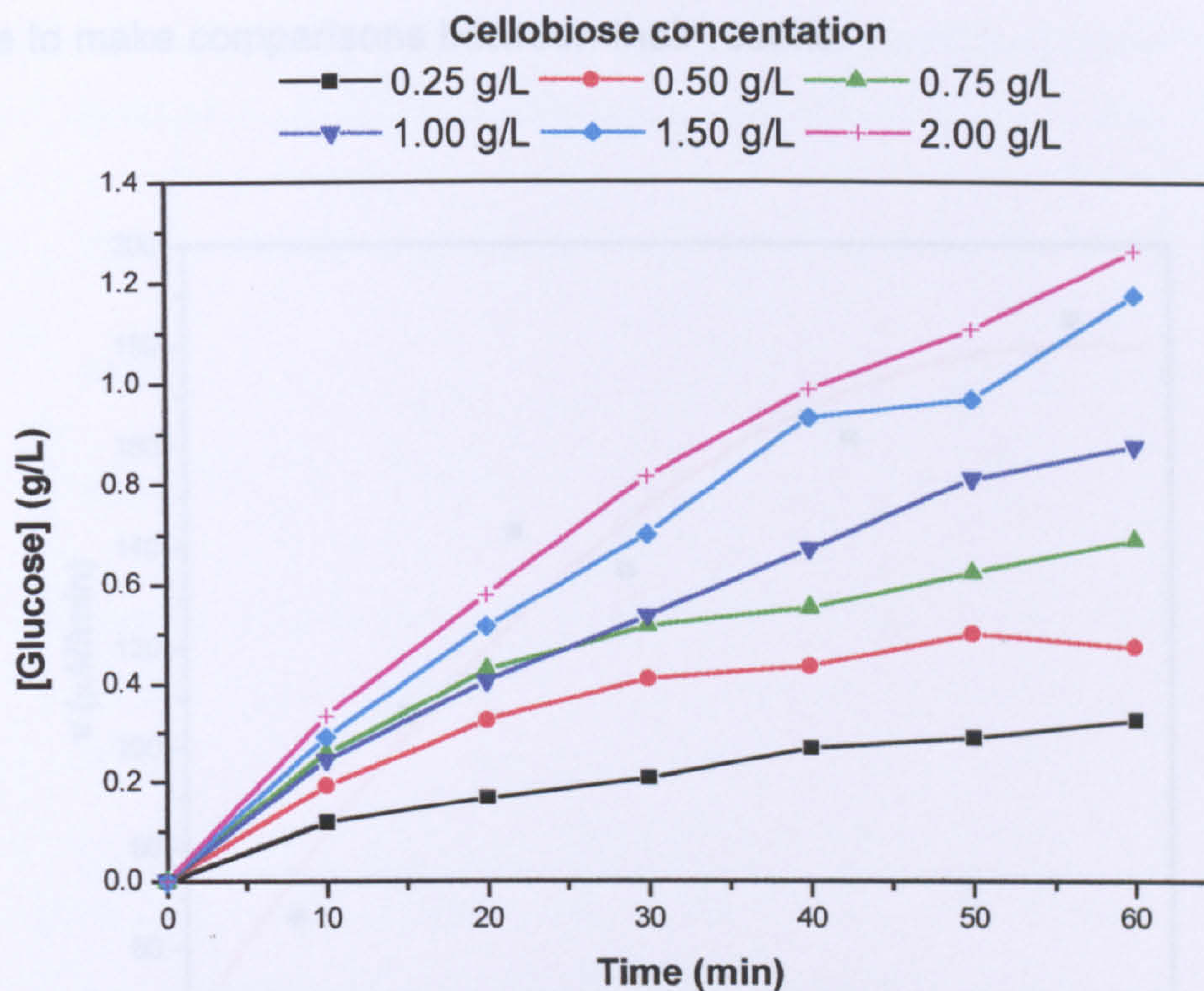


Figure 4.7 Conversion of cellobiose to glucose by the cellobiase present in TC of *T. reesei* (0.16 mg total protein/L, 50 °C, pH 5.0), using various concentrations of cellobiose (0.25-2.00 g/L).

From Figure 4.9 it is possible to obtain a linear relation between 1/v and 1/[S] with a correlation coefficient 0.992. Thus the Michaelis-Menten model fits well the degradation of cellobiose by β-glucosidase from *T. reesei* cellulase crude, in the range of concentrations studied (0.25-2 g/L, 0.73-5.85 mM). The parameters obtained from the Lineweaver-Burk equation were 242.23 μM/min and 1.91 mM (0.653 g/L) for v_{max} and K_m respectively. Since the protein concentration of β-glucosidase in the cellulase mixture is unknown, it is not possible to express the v_{max} value in terms of mg of protein. Several other groups of workers (Calsavara *et al.* 1999; Dekker, 1986; Lee and Fan, 1983) have published values for v_{max} and K_m but these authours used different

methodologies and models to determine these parameters. It is therefore not possible to make comparisons between their results.

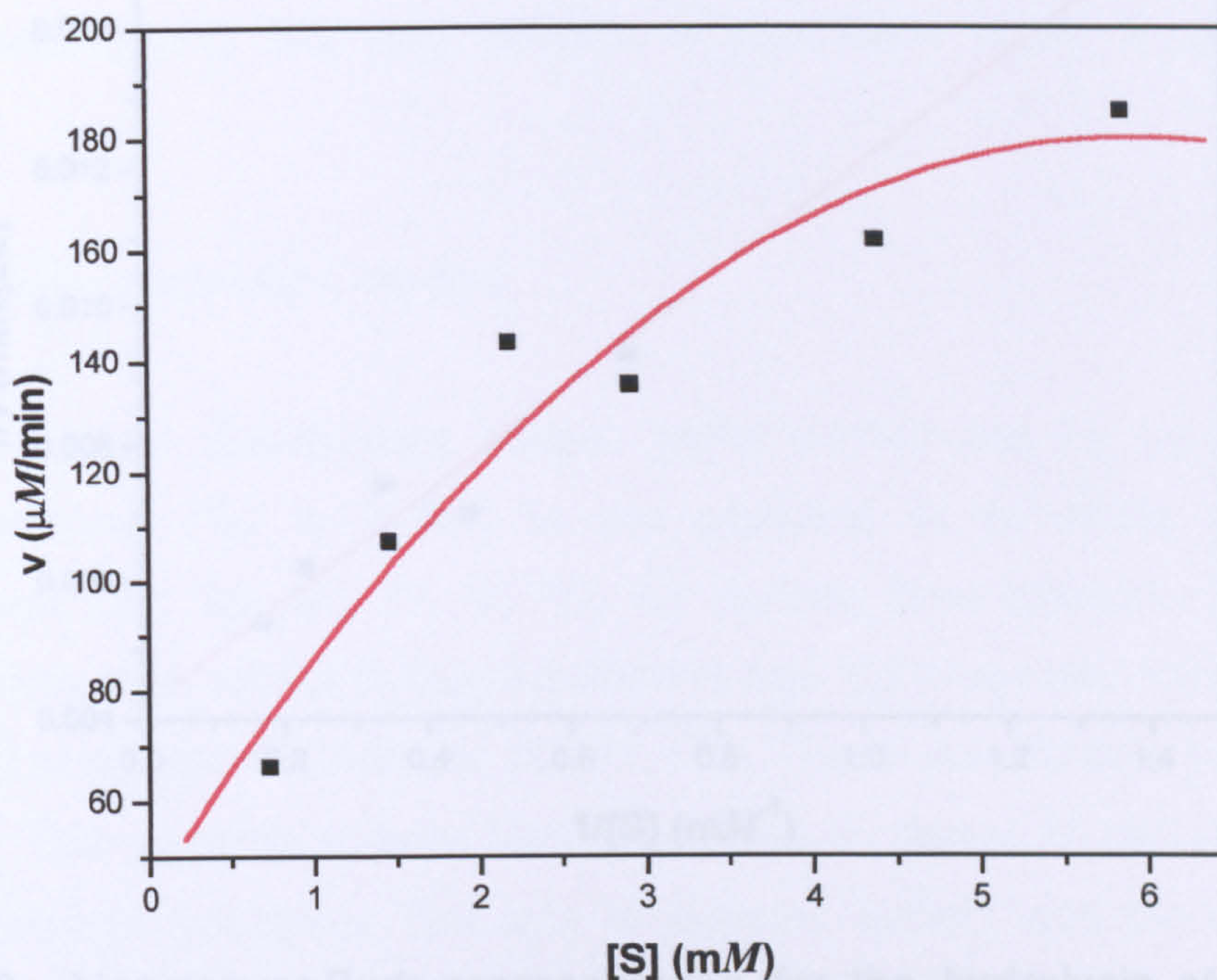


Figure 4.8 Glucose formation rate using cellobiose as substrate (50 ºC, pH 5.0, 10 min) for TC of *T. reesei*.

The activity at 50 ºC and pH 5.0 after 60 min of incubation, of the cellulase mixture with 2.0 g/L of cellobiose, was found to be 0.76 U/mg total protein, which is very low compared with other cellobiase activities given in the literature. Dekker (1986) and Calsavara *et al.* (1999) found maximum activities on cellobiose, for isolated β-glucosidase from *Aspergillus niger*, of 251 U/mL (50 ºC, pH 5.0) and 17.84 U/mg protein (65 ºC, pH 4.5) respectively. It is therefore important to supplement the cellulase mixture with exogenous cellobiase activity to increase the degradation of cellobiose produced during the enzymatic treatment and thereby decrease its inhibition of the cellulases.

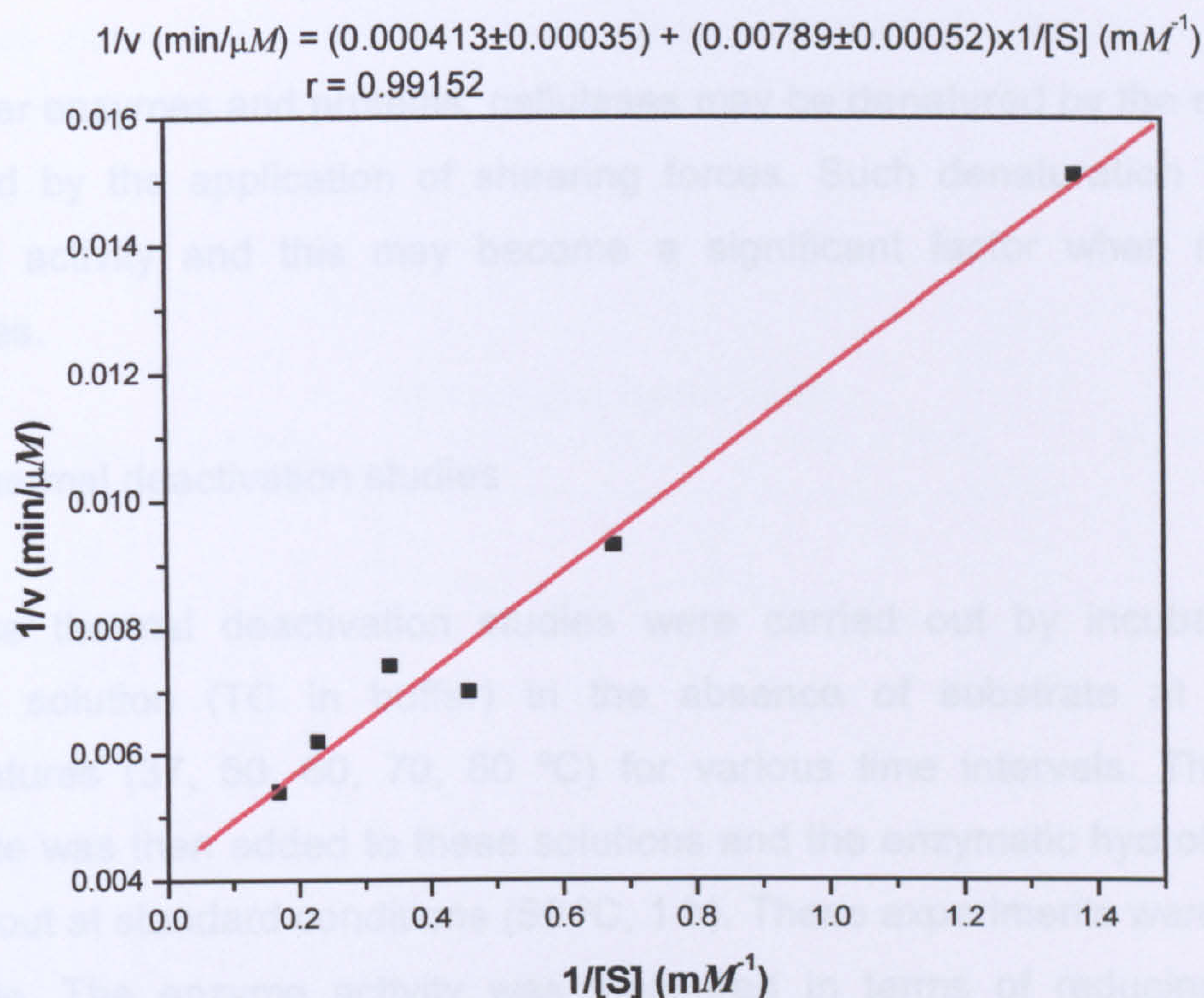


Figure 4.9 Lineweaver-Burk representation for the hydrolysis of cellobiose (0.16 mg total protein/L, 50 ºC, pH 5.0, 10 min) by the cellobiase present in a crude mixture of *T. reesei* cellulase.

It is also known that β-glucosidase is inhibited by glucose. Several authors (Calsavara *et al.*, 1999; Dekker, 1986; Lee and Fan, 1983) have studied this phenomenon and found that glucose inhibits β-glucosidase through a fully or partially competitive mechanism. Dekker (1986) reported that *Aspergillus niger* β-glucosidase was remarkably active in the presence of high amounts of glucose; he showed that at 30 g/L of glucose the β-glucosidase retained 72% of its activity in the absence of glucose. Calsavara *et al.* (1999) found that the presence of glucose (1.8 g/L) retarded the enzymatic conversion of cellobiose. Lee and Fan (1983) found a value of 1.32 g/L (7.3 m*M*) of glucose for the inhibition constant for β-glucosidase. The removal of glucose from process liquors is therefore also important when recycling is considered.

4.3 Thermal and mechanical deactivation of cellulases

Like other enzymes and proteins, cellulases may be denatured by the effects of heat and by the application of shearing forces. Such denaturation leads to reduced activity and this may become a significant factor when recycling cellulases.

4.3.1 Thermal deactivation studies

Cellulase thermal deactivation studies were carried out by incubating the enzyme solution (TC in buffer) in the absence of substrate at different temperatures (37, 50, 60, 70, 80 °C) for various time intervals. The cotton substrate was then added to these solutions and the enzymatic hydrolysis was carried out at standard conditions (50 °C, 1 h). These experiments were done in duplicate. The enzyme activity was measured in terms of reducing sugars released during hydrolysis. The loss of enzyme activity was calculated as a percentage of the activity of the enzyme solution without thermal pretreatment. Enzyme adsorbed on the fabrics was also measured (see 3.2.5) after the thermal treatments. The results are listed in Tables II and III in Appendix A and are illustrated in Figures 4.10 and 4.11.

From Figure 4.10 it may be seen that at 60 °C the cellulase system lost much of its activity and the treatment time enhanced this effect. At 70 and 80 °C, the enzyme was completely denatured and did not show any activity. Comparing the temperature effects at 37 and 50 °C, it seems that using more moderate temperatures gives improved enzyme stability. The activity after incubation at 37 °C was found to increase by 10-20% and this may be explained by the increased adsorption of the incubated enzymes (Figure 4.11). It is also apparent that cellulases lose some of their activity at 50 °C after long incubation times. This should be considered when repeated use of the enzymes is desired and the activity decay should be predicted and compensated for by the addition of fresh cellulase. It should be noted that in these thermal deactivation studies

the thermal treatments were carried out in the absence of cotton fabric. Some substrates have been reported to be enzyme stabilizers (Bayley and Ollis, 1987) so the observed deactivation effects may be different during actual fabric treatments.

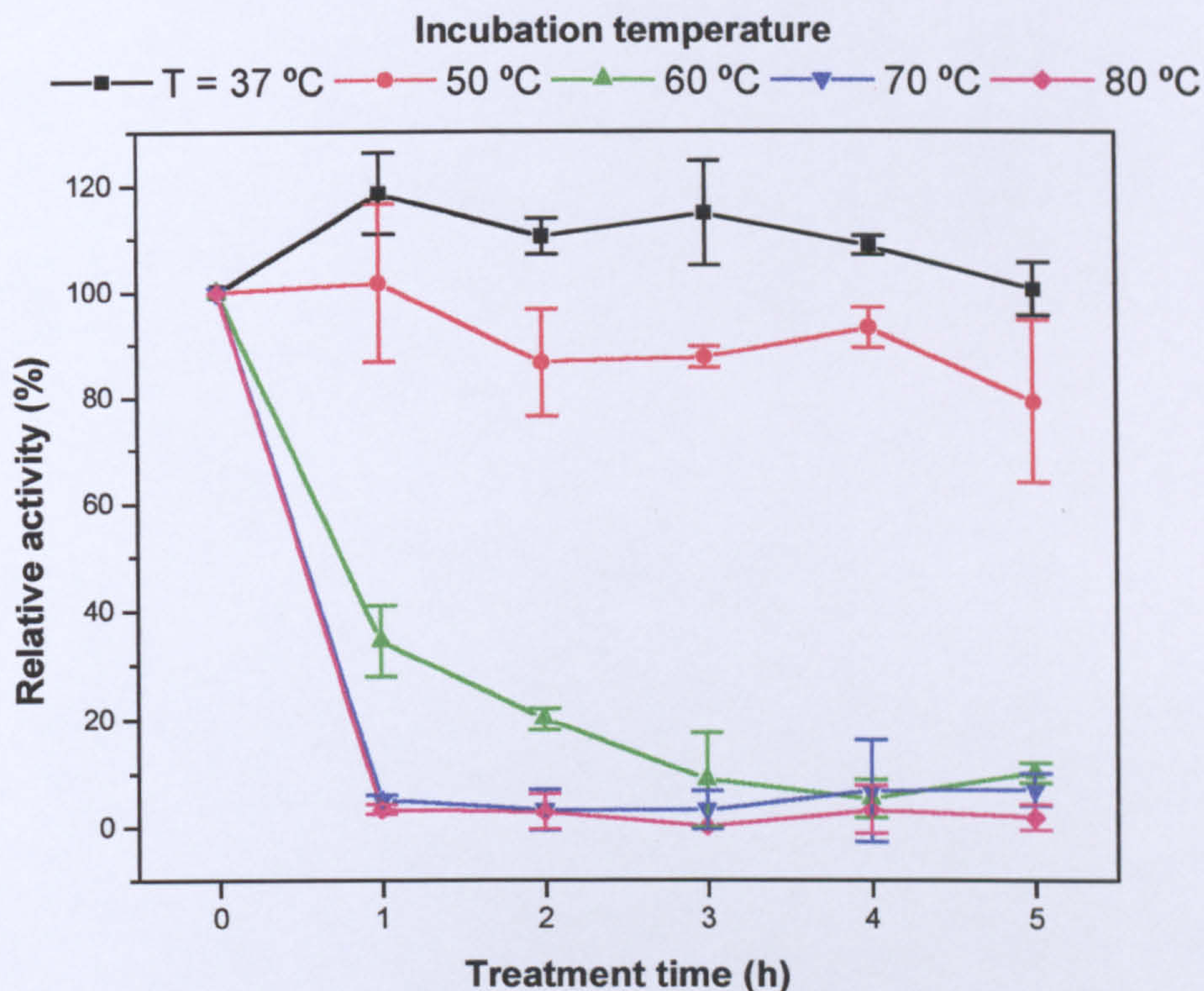


Figure 4.10 Effect of thermal pretreatment on cellulase (TC) activity (measured in terms of reducing sugars produced during hydrolysis of cotton fabrics at 50 ºC, pH 5.0, 1 h) relative to the activity of cellulase without thermal pre-treatment. The values in the figure represent the means of two independent experiments and error bars the standard deviation.

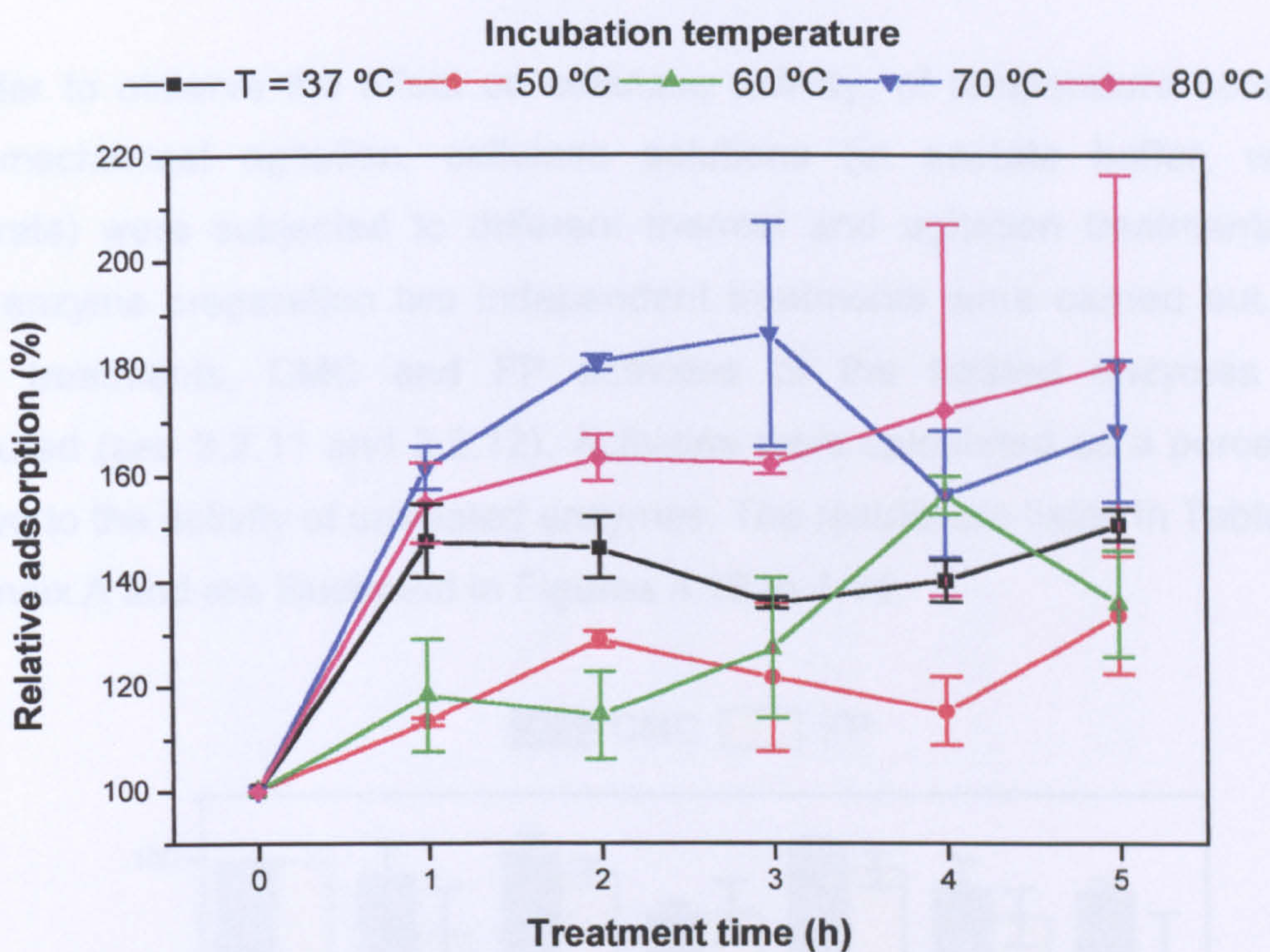


Figure 4.11 Effect of thermal pretreatment on cellulase (TC) adsorption (measured in terms of protein adsorbed on fabrics after hydrolysis of cotton fabrics at 50 ºC, pH 5.0, 1 h) relative to adsorption from the cellulase solution without thermal pre-treatment. The values in the figure represent the means of two independent experiments and error bars the standard deviation.

Whereas the catalytic activity of cellulases is completely lost at higher temperatures, the binding capacity of the protein on cotton fabrics (Figure 4.11) seems to increase. This may be due to the exposure of new aromatic residues on the protein surface (caused by the unfolding of the protein after denaturation) which would contribute to an increase in the hydrophobic interactions between the protein and the cotton fabric. The possibility that increasing amounts of denatured cellulase rather than the active enzyme may be more strongly bound to cellulose should therefore be considered when cellulases are to be reused over many cycles.

4.3.2 Thermal and mechanical treatments of enzyme solutions

In order to observe the effect on cellulase activity, of temperature combined with mechanical agitation, cellulase solutions (in acetate buffer, without substrate) were subjected to different thermal and agitation treatments. For each enzyme preparation two independent treatments were carried out. After these treatments, CMC and FP activities of the treated enzymes were measured (see 3.2.11 and 3.2.12). Activities were calculated as a percentage relative to the activity of untreated enzymes. The results are listed in Table IV in Appendix A and are illustrated in Figures 4.12 to 4.14.

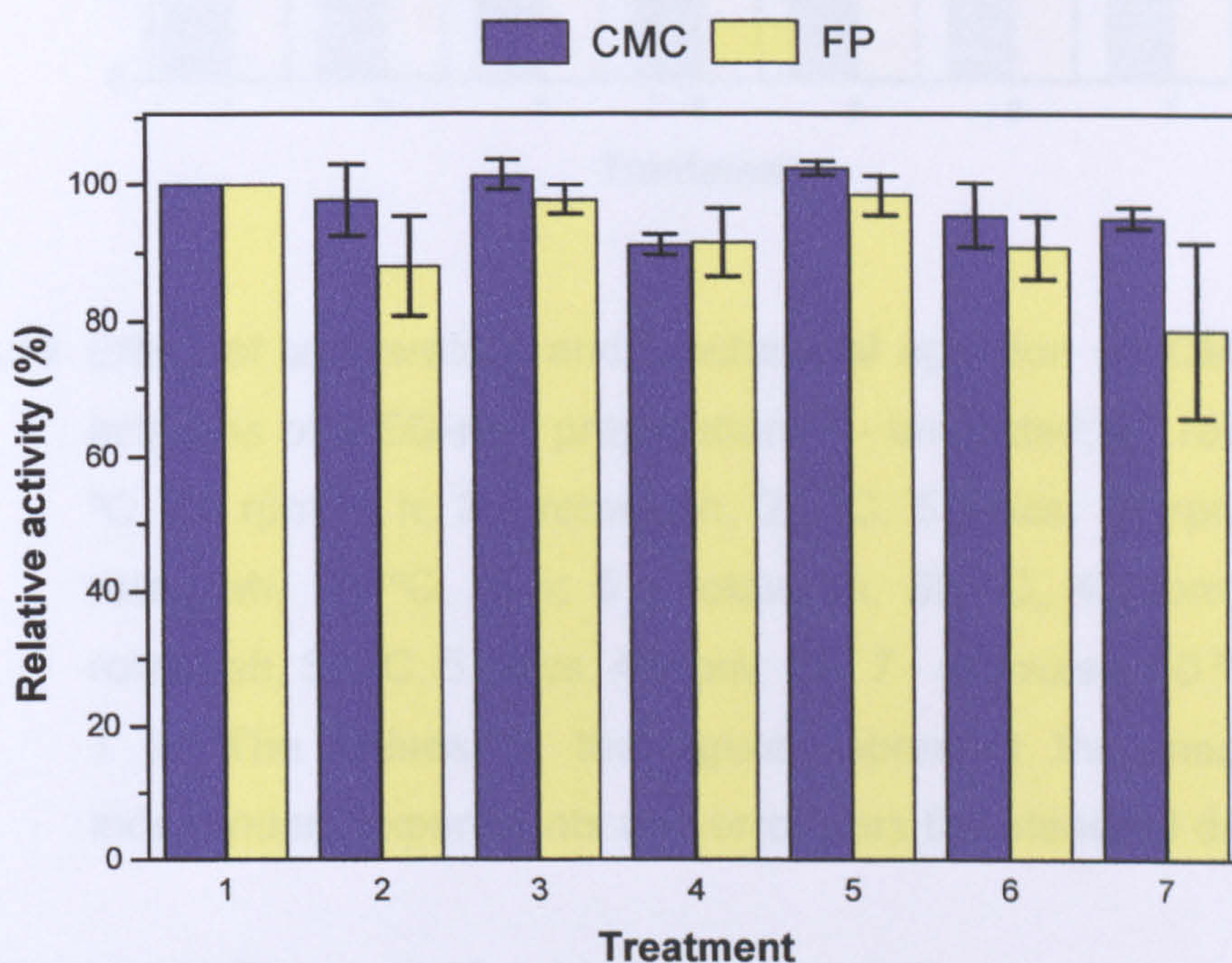


Figure 4.12 Effect of temperature and mechanical agitation on CMC and FP activities of a total cellulase (**TC**). **1** - untreated; **2** - rotawash, 22 °C, 40 rpm, 1 h; **3** - rotawash, 22 °C, 5 discs, 40 rpm, 1 h; **4** - rotawash, 50 °C, 1 h; **5** - rotawash, 50 °C, 40 rpm, 1 h; **6** - rotawash, 50 °C, 5 discs, 40 rpm, 1 h; **7** - rotawash, 60 °C, 40 rpm, 1 h. The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

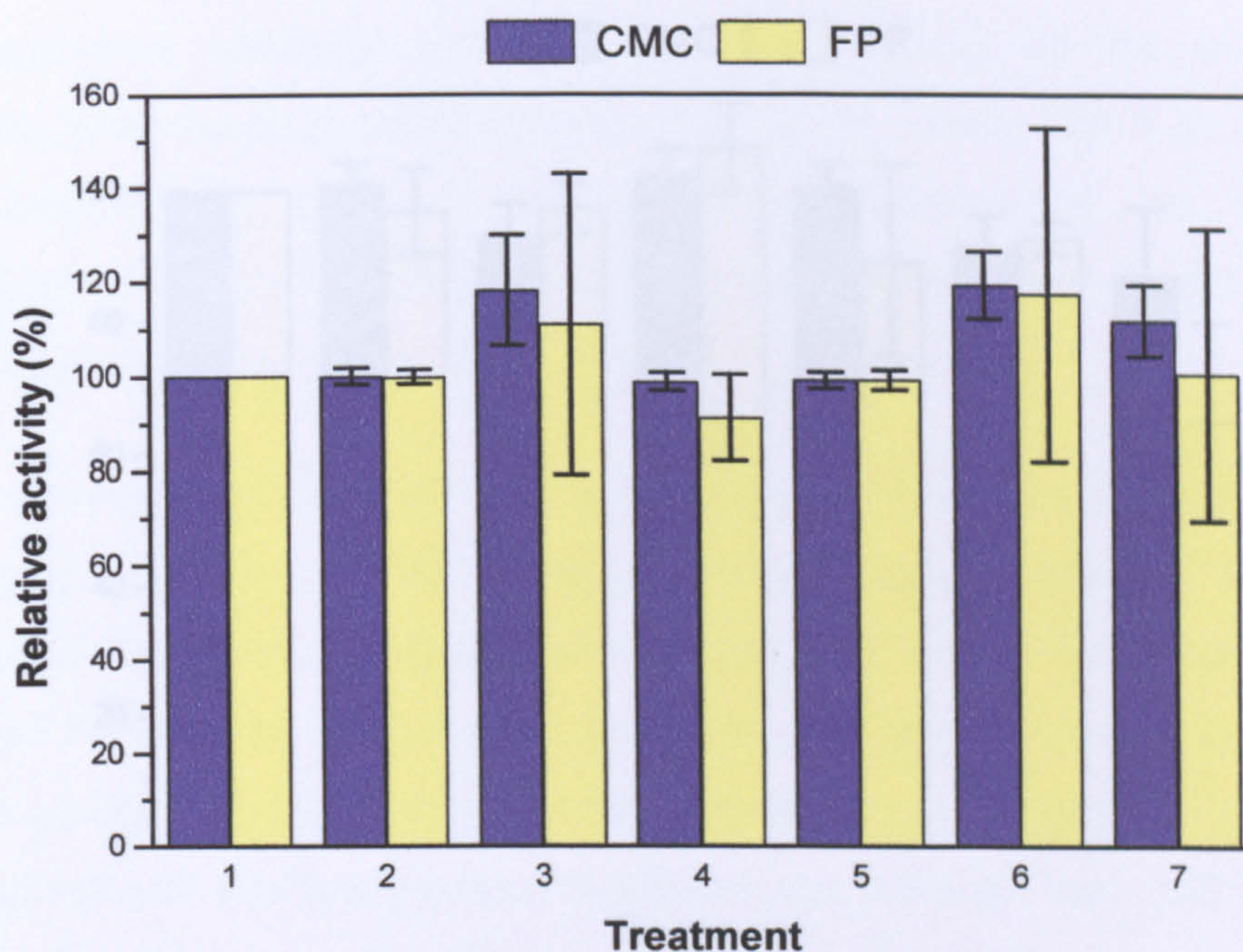


Figure 4.13 Effect of temperature and mechanical agitation on CMC and FP activities of a **EG-rich** preparation. **1** - untreated; **2** - rotawash, 22 ºC, 40 rpm, 1 h; **3** - rotawash, 22 ºC, 5 discs, 40 rpm, 1 h; **4** - rotawash, 50 ºC, 1 h; **5** - rotawash, 50 ºC, 40 rpm, 1 h; **6** - rotawash, 50 ºC, 5 discs, 40 rpm, 1 h; **7** - rotawash, 60 ºC, 40 rpm, 1 h. The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

The results given in Figures 4.12-4.14 show that agitation levels typical of textile processing do not cause significant mechanical deactivation. Treatments at 60ºC caused partial deactivation which was more apparent in FP activity and for the CBH-rich composition. This suggests that CBHs are less thermally stable than EGs. More information about the effects of temperature on protein conformation can, however be obtained using fluorescence emission spectroscopy.

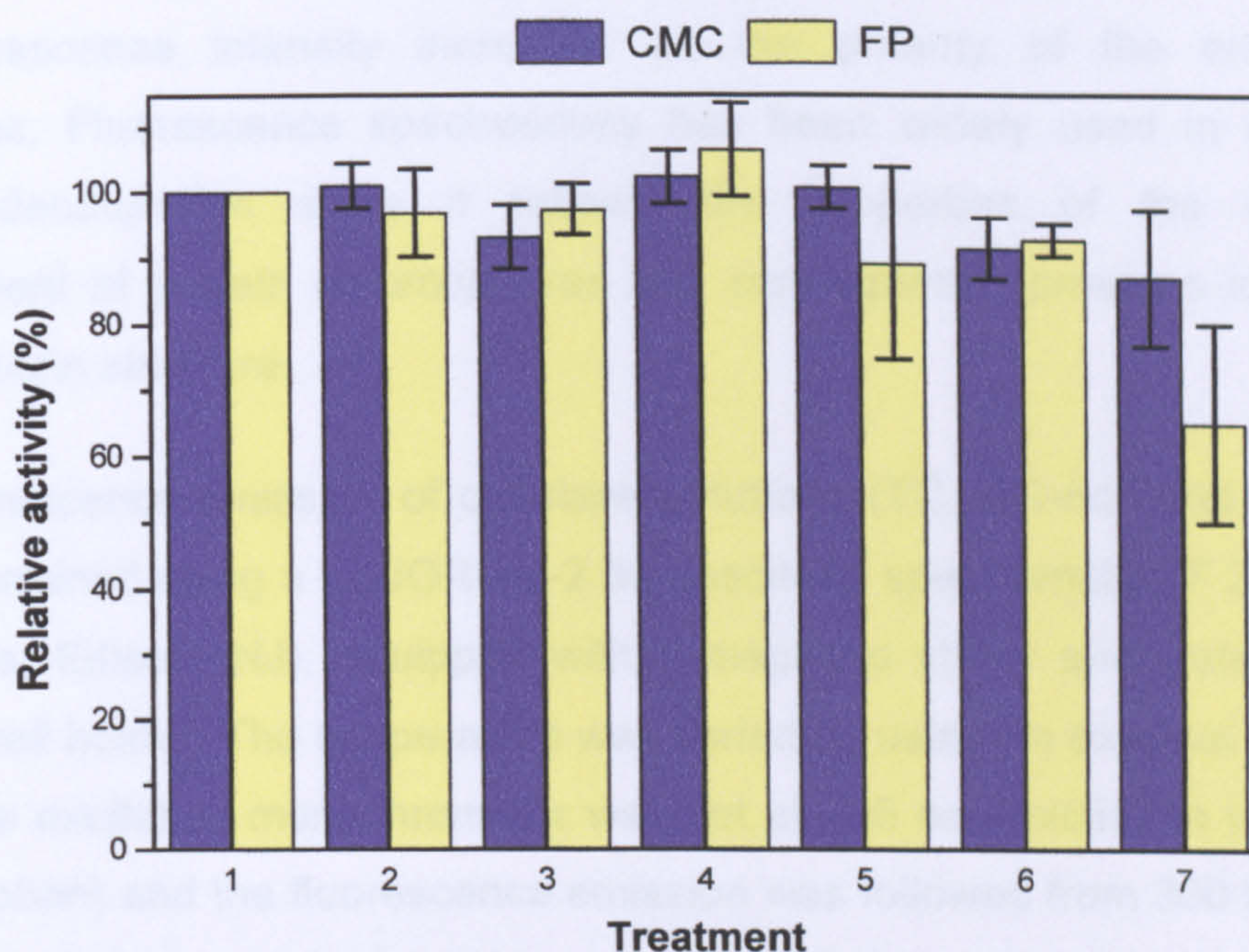


Figure 4.14 Effect of temperature and mechanical agitation on CMC and FP activities of a **CBH-rich** preparation. **1** - untreated; **2** - rotawash, 22 ºC, 40 rpm, 1 h; **3** - rotawash, 22 ºC, 5 discs, 40 rpm, 1 h; **4** - rotawash, 50 ºC, 1 h; **5** - rotawash, 50 ºC, 40 rpm, 1 h; **6** - rotawash, 50 ºC, 5 discs, 40 rpm, 1 h; **7** - rotawash, 60 ºC, 40 rpm, 1 h. The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

4.3.3 Conformational changes in protein structure caused by heating, as measured by fluorescence emission spectroscopy

Proteins contain aromatic amino acids such as tryptophan, tyrosine and phenylalanine, which exhibit fluorescence. Tryptophan is the strongest fluorescer with excitation at 280-285 nm and fluorescence near 350 nm (Creagh *et al.*, 1993; Permyakov, 1993). Fluorescence is sensitive to the environment of the fluorescer and both the wavelength of maximum emission (λ_{max}) and the fluorescence intensity (quantum yield) may be affected. Usually a shift in λ_{max}

indicates a change in polarity of the environment of the fluorescer. In general, the fluorescence intensity increases as the polarity of the environment decreases. Fluorescence spectroscopy has been widely used in studies of protein denaturation since it reflects the properties of the immediate environment of protein chromophores and consequently provides information about protein structure.

The fluorescence emission of cellulase solutions (TC, EG-rich and CBH-rich) was determined using a FLUOROG-2 fluorescence spectrometer (F 2127, Spex Industries, Edison, NJ), equipped with a magnetic stirrer and water-jacketed sample cell holder. The temperature was varied by using an external circulating bath. The excitation monochromator was set at 285 nm (excitation wavelength of tryptophan) and the fluorescence emission was followed from 300 to 450 nm. Maximum emission wavelength was recorded for each sample over the temperature range 20-80 °C.

The wavelength of maximum fluorescence emission (λ_{max}) by tryptophan residues in proteins, varies within the limits of 307 to 353 nm and depends on the location of the residue in the protein globular structure (inside the molecule or at the surface). Permyakov (1993) found that thermal denaturation of tryptophan-containing proteins usually results in a pronounced shift of the tryptophan fluorescence spectrum to longer wavelengths.
Figure 4.15 shows the temperature dependence of λ_{max} for the three cellulase preparations. Since these preparations are not purified single cellulolytic components, it is impossible to know which component in the mixture is responsible for a particular effect. Furthermore, since each cellulase component possesses multiple tryptophan residues located in different parts of the molecule, it is also impossible to know which tryptophan residue is responsible for the shift in the wavelength of emission. Nevertheless it can be seen from Figure 4.15 that the three cellulase crudes showed different behaviours over the temperature range studied. CBH-rich showed a slight shift in λ_{max} to shorter wavelength between 20 and 40 °C and then a progressive shift to longer

wavelength between 40 and 80 °C. EG-rich, in contrast, showed an initial shift in λ_{max} to longer wavelength between 20 and 50 °C followed by a very sharp shift to shorter wavelength at 60 °C and then a marked shift to longer wavelength between 60 and 80 °C. TC being a mixture of these EGs and CBHs showed intermediate behaviour with a minimum in λ_{max} at 60 °C reflecting the EG behaviour more than that of the CBHs.

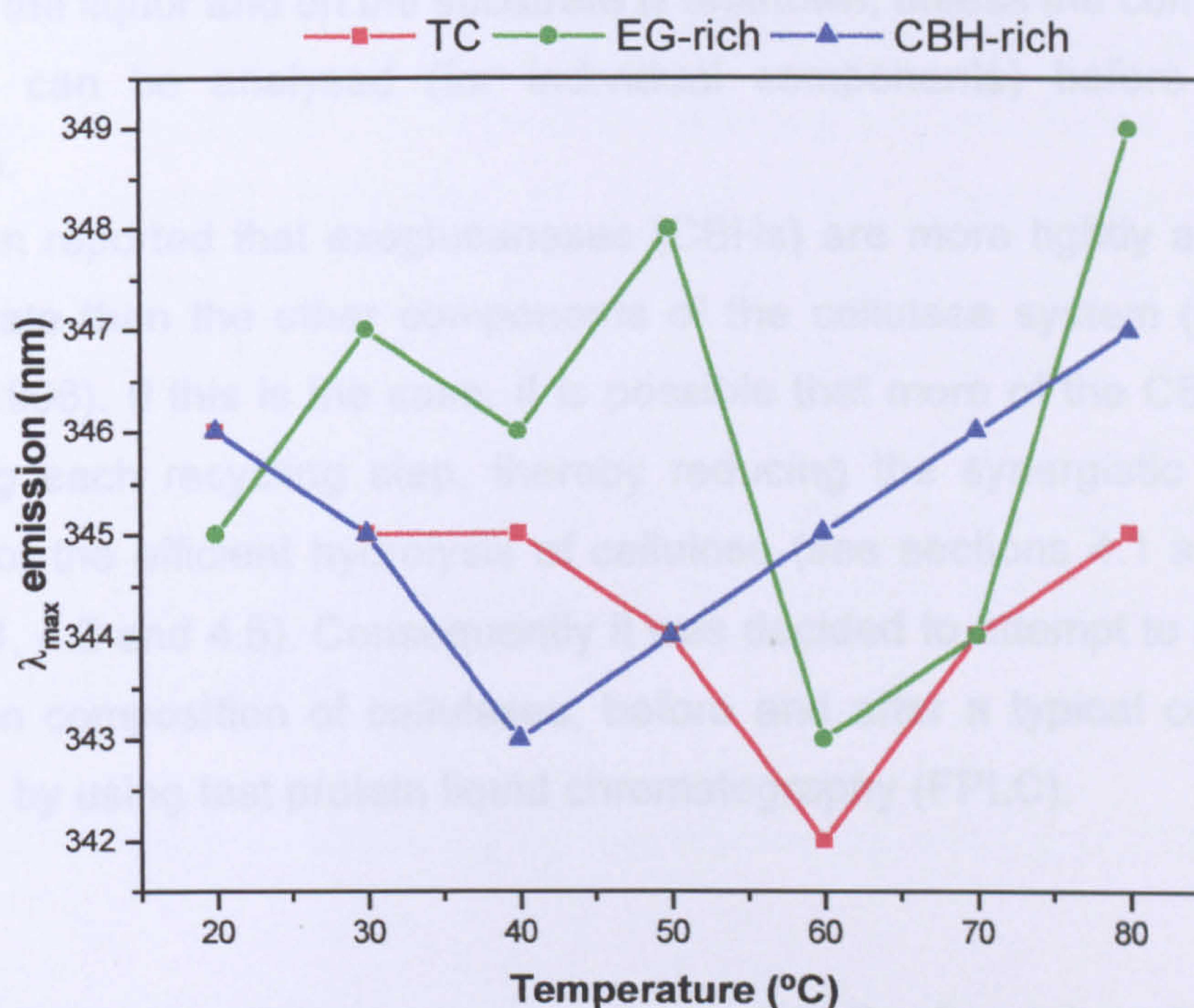


Figure 4.15 Temperature dependence of the wavelength of maximum fluorescence emission for the three cellulase preparations.

The denaturation of a protein is usually followed by its coagulation and precipitation. It was possible to observe that CBH-rich solutions became more turbid compared with EG-rich solutions after they had been subject to a 50 °C (1 h) thermal treatment and this seems to be consistent with the progressive shift in λ_{max} of the fluorescence emission to longer wavelengths above 40 °C. This is consistent with the results shown in Figures 4.12-4.14 and also with the findings

of Baker *et al.* (1992) who found that the EGII of *T. reesei* is more thermally stable (by about 10 °C) than the other main components of *T. reesei* cellulases.

4.4 Adsorption and desorption of various cellulases and their components

The cellulases used in textile processing are usually mixtures of components having different adsorptions and activities. The portion of each component present in the liquor and on the substrate is unknown, unless the composition of the liquor can be analysed (for individual components) before and after adsorption.

It has been reported that exoglucanases (CBHs) are more tightly adsorbed to the substrate than the other components of the cellulase system (Gregg and Saddler, 1996). If this is the case, it is possible that more of the CBHs can be lost during each recycling step, thereby reducing the synergistic interaction required for the efficient hydrolysis of cellulose (see sections 4.1 and 4.2 and Tables 4.1, 4.2 and 4.5). Consequently it was decided to attempt to analyse for changes in composition of cellulases, before and after a typical cotton fabric treatment, by using fast protein liquid chromatography (FPLC).

4.4.1 Analysis of cellulase components by FPLC after adsorption on and hydrolysis of cotton fabric

Before use in these experiments cellulase solutions (in sodium acetate buffer, 0.1 *M*, pH 5.0) were ultrafiltered through a 0.09 m^2 ultrafiltration cartridge with a Molecular Weight Cut-Off (MWCO) of 10 kDa (Prep/Scale TFF-1, Millipore Corporation, Bedford, MA, USA).

Adsorption and hydrolysis experiments on cotton fabrics

The adsorption and hydrolysis treatments were performed in a Rotawash machine for 1 hour at 50 °C and 40 rpm. Samples of cotton fabric (7 g) were placed in the in stainless steel pots (500 mL) with 100 mL of a cellulase solution in sodium acetate buffer (0.1 *M*, pH 5.0). Liquor samples were taken to measure the total protein present in solution and for protein analysis by FPLC. The treatment liquors (hydrolysates) were centrifuged (10 min, 2875*g*) to remove insoluble material (cotton powder). The supernatant was analysed for total protein determination, total soluble reducing sugars. Fabric weight loss was also determined according to 3.2.10. The supernatants were then ultrafiltered (as described above) to remove the soluble reducing sugars. The protein adsorbed by the fibre was determined as the difference between initial protein concentration and the concentration of the protein remaining in the supernatant. FPLC analysis of the ultrafiltered supernatant was performed to determine the change in composition of the cellulase complex remaining in solution after cotton treatment.

Analysis of cellulase components by fast chromatofocusing (FPLC)

For this technique, a pH range was chosen so that the isoelectic points (pIs) of the proteins of interest fell roughly in the middle of the pH gradient. An appropriate column exchanger was equilibrated with the start buffer, with a pH set slightly above the upper limit of the pH gradient. The pH of the eluent was adjusted to the value chosen for the lower limit of the pH gradient. The sample was equilibrated with the eluent and applied to the column. The elution was then carried out with a special buffer (Polybuffer™, which contains a large number of buffering species to give even buffering capacity) and the pH gradient formed automatically. Proteins eluted in order of their isolectric points. The charge on a protein depends on the pH and the pI of the protein. When the pH is less than its pI, a protein carries a positive charge and will migrate down

the column of anion exchanger in the eluting buffer. As it migrates, however, the pH of the buffer surrounding the protein increases with the distance from the top of the column. When it has travelled sufficiently far down the column so that the pH is greater than pI, the protein reverses its charge and binds to the ion exchanger. The molecule remains bound to the ion exchanger until the developing pH gradient causes the pH to drop below its pI. The protein is then carried along in the eluent buffer again until the pH rises above the pI and it rebinds. This process is repeated until the protein emerges from the column at its pI.

In the present work, column equilibration was performed with the start buffer (25 mM Bis-Tris adjusted to pH 6.9 with hydrochloric acid (2 M) supplemented with 10% of betaine monohydrate) until the column effluent was at the same pH as the start buffer. Cellulase samples diluted in start buffer were filtered through a small syringe filter (Millex-GV13, diameter 13 mm, sterile, low protein binding, polyvinylidene difluoride filter membrane, pore size 0.22 µm, Millipore, Bedford, MA, USA) before being applied to a Mono P HR 5/20 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The sample was injected by first running 5 mL of eluent to avoid protein exposure to extremes of pH. In some cases, 0.5-4 mL centrifugal ultrafiltration units (Ultrafree-4, Millipore Corporation, Bedford, MA, USA) with 5000 MWCO, polyethersulphone membranes, were used to concentrate the samples before injection. The elution was then carried out at 1 mL/min with 37 mL of 1:10 diluted Polybuffer 74 (Amersham Pharmacia Biotech, Uppsala, Sweden) adjusted to pH 4.0 with hydrochloric acid (2 M) supplemented with 10% of betaine monohydrate, followed by a further 43 mL of 1:10 diluted Polybuffer 74 adjusted to pH 3.0 with hydrochloric acid (2 M) also supplemented with 10% of betaine monohydrate. All eluents and cleaning solutions were continuously vacuum degassed before entering the column. The protein detection was monitored using a UV detector (Welchrom K-2500, Knauer, Berlin, Germany) set to 280 nm. The pH of the column effluent was also monitored and recorded to check the formation of the pH gradient and to observe the elution pH of protein fractions. Fractions of the

column effluent were collected and were used for determination of protein content and measurement of CMC and FP activity. Protein sample loads usually varied from 0.1 to 1 mg but when fractions were to be collected 1-2 mg of protein was applied.

The FPLC equipment was purchased especially for this project. Consequently a long learning and optimisation process was necessary before reproducible results (see chromatograms in Appendix B) could be obtained. The number of analyses completed was therefore limited. In further work it would be interesting to examine the cellulase composition after multiple recycling steps as well as after one fabric treatment and also to analyse the composition of cellulase components desorbed from the treated fabrics.

Table 4.7 Characterization of cellulase preparations in terms of protein adsorption and activity (weight loss and production of reducing sugars) on cotton fabrics (Rotawash machine, 50 °C, 40 rpm, 1 h, pH 5.0, 9 mg protein/g fabric, 1:14 liquor ratio). Values in brackets represent the standard deviation of two independent experiments.

Cellulase preparation	Protein adsorption (%)	Weight loss (%)	[Reducing sugars] (g/L)
TC	25.8 (3.1)	4.45 (0.58)	1.60
EG-rich	11.9 (0.6)	1.38 (0.33)	1.35 (0.03)
CBH-rich	18.7 (0.8)	3.02 (0.26)	1.69 (0.06)

In Table 4.7, some results of protein adsorption and activity on cotton fabrics are given for the different cellulase compositions. With respect to the adsorption, it seems that CBHs adsorb more on cotton than EGs and if all components are present (TC preparation) more enzyme is adsorbed, revealing some synergism in the adsorption. The complete system caused the most fabric

weight loss followed by the CBH-rich mixture and EG-rich produced the least weight loss.

The identification of the cellulase components on the chromatograms was made considering their pIs (Table 2.2, section 2.1.3), their percentage in the preparation (Table 3.1, section 3.2.3) and the activities on CMC and FP of the fractions collected. Since, in the chromatofocusing technique, the elution of the proteins is in order of decreasing isoelectric points, the first peak to be eluted corresponds to proteins which have pIs greater than pH 6.9 (equilibration pH with the start buffer) and which are therefore not adsorbed to the column. Under these conditions, when a cellulolytic system of *T. reesei* is analysed, it is expected that β-glucosidase (pI 8.7) will elute with or near the solvent front. Then CBHII (pI 5.1-6.3) or EGII, (pI 5.5) will appear followed by EGI (pI 4.6) and CBHI (pI 3.5-4.2). It should be noted that with this chromatofocusing technique the elution time may vary, and it is the elution pH which characterises specific protein peaks. In Figures 4.16-4.18 the chromatographic profiles of the three cellulase preparations from *T. reesei* are illustrated. The complete fractionation requires about 80 minutes and TC mixture could be separated into 13 distinct peaks (Figure 4.19). This is in agreement with the findings of other authors who have analysed *T. reesei* cellulase mixtures using the same technique. Hayne and Esterbauer (1985) obtained 14 fractions and Yu *et al.* (1993) separated a commercial cellulase preparation into 15 components. Ellouz *et al.* (1987) used ion-exchange FPLC for analysing *T. reesei* cellulases and obtained 14 distinct peaks.

The main components in the cellulolytic mixture of *T. reesei* are CBHI, CBHII, EGI and EGII, the other peaks probably represent isoforms of the components (see below), minor EGs, and other non-cellulase proteins.

At first, it was thought that the last peak (12 and 13) in the TC chromatogram (Figure 4.19) could represent CBHI because it is a very significant peak and it was eluted at pH ≈ 3.8, which is in the pI interval (3.5-4.2) for this protein. This

peak, however, also appeared in the EG-rich preparation from which the CBHI gene was deleted (Figure 4.17).

Fractions of the sample analysed in Figure 4.19 were collected for determination of CMC and FP activity. Peaks number 12 and 13 did not show any CMC or FP activity and these therefore probably contained a protease since it is known that, depending on the culture medium, *T. reesei* produces, in addition to cellulases, various side activities including an aspartic protease (pI 3.8).

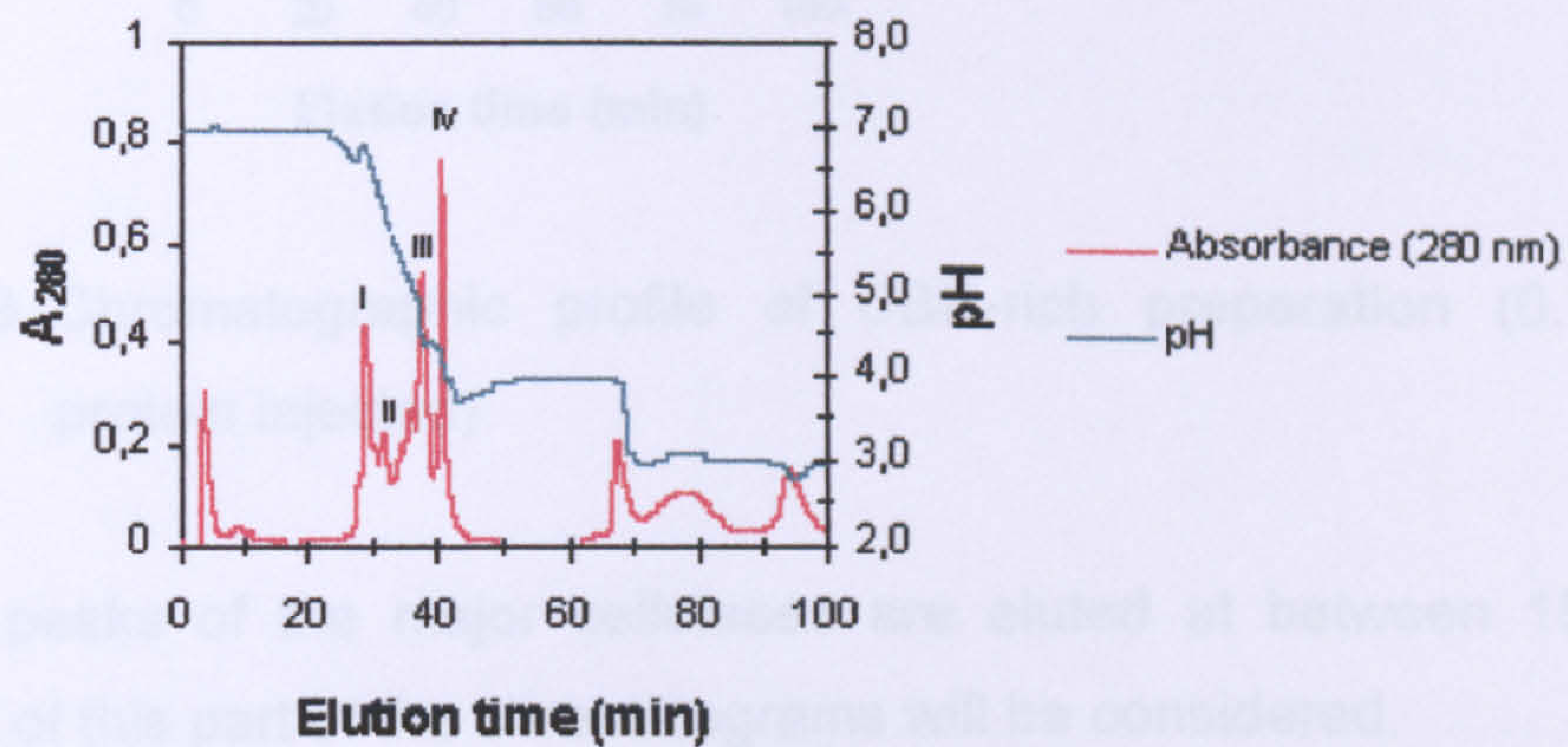


Figure 4.16 Chromatographic profile of TC preparation (1 mg of protein injected). Roman numerals in the chromatograph represent CBHII, EGII, EGI and CBHI respectively.

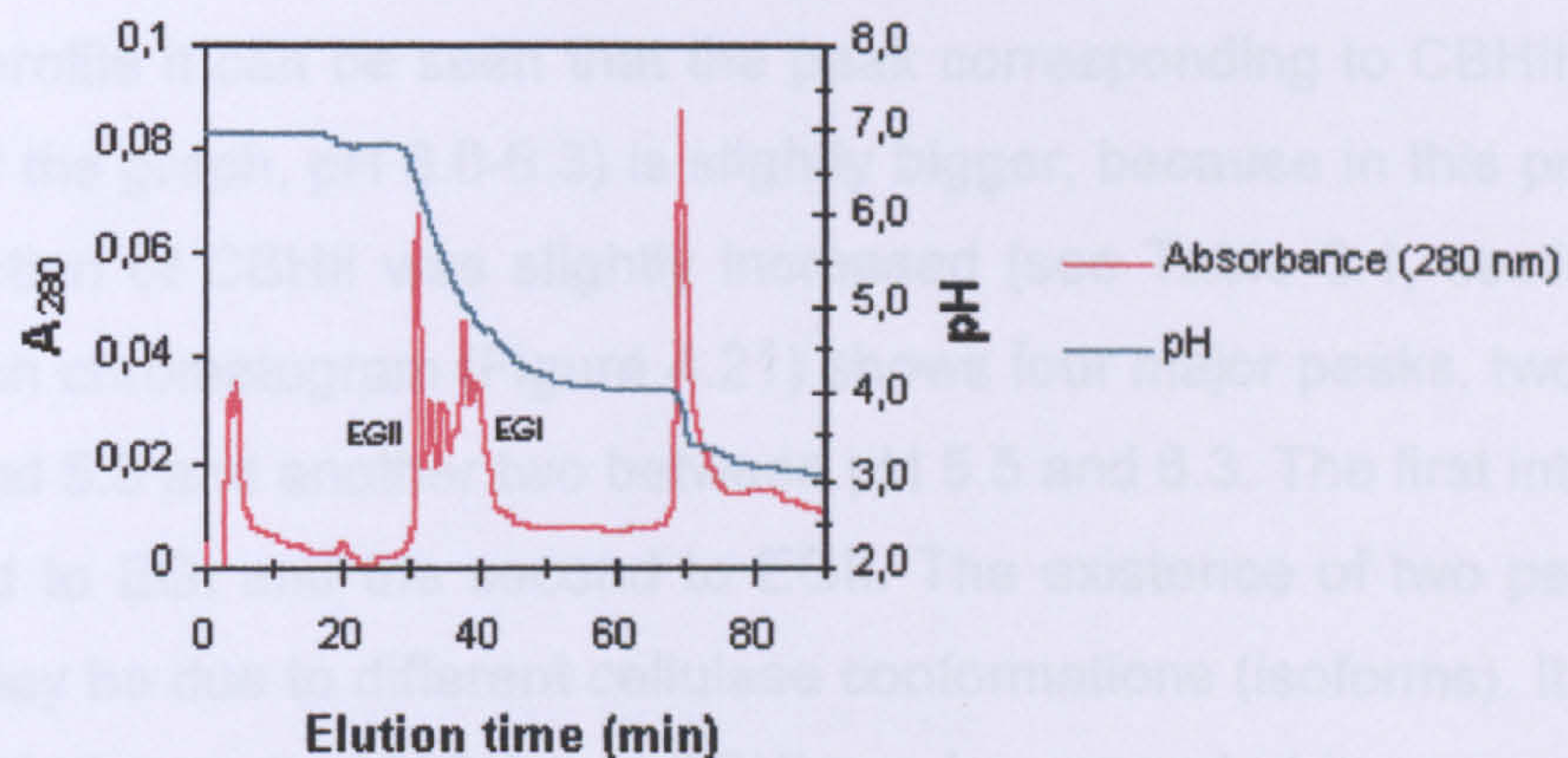


Figure 4.17 Chromatographic profile of EG-rich preparation (0.1 mg of protein injected).

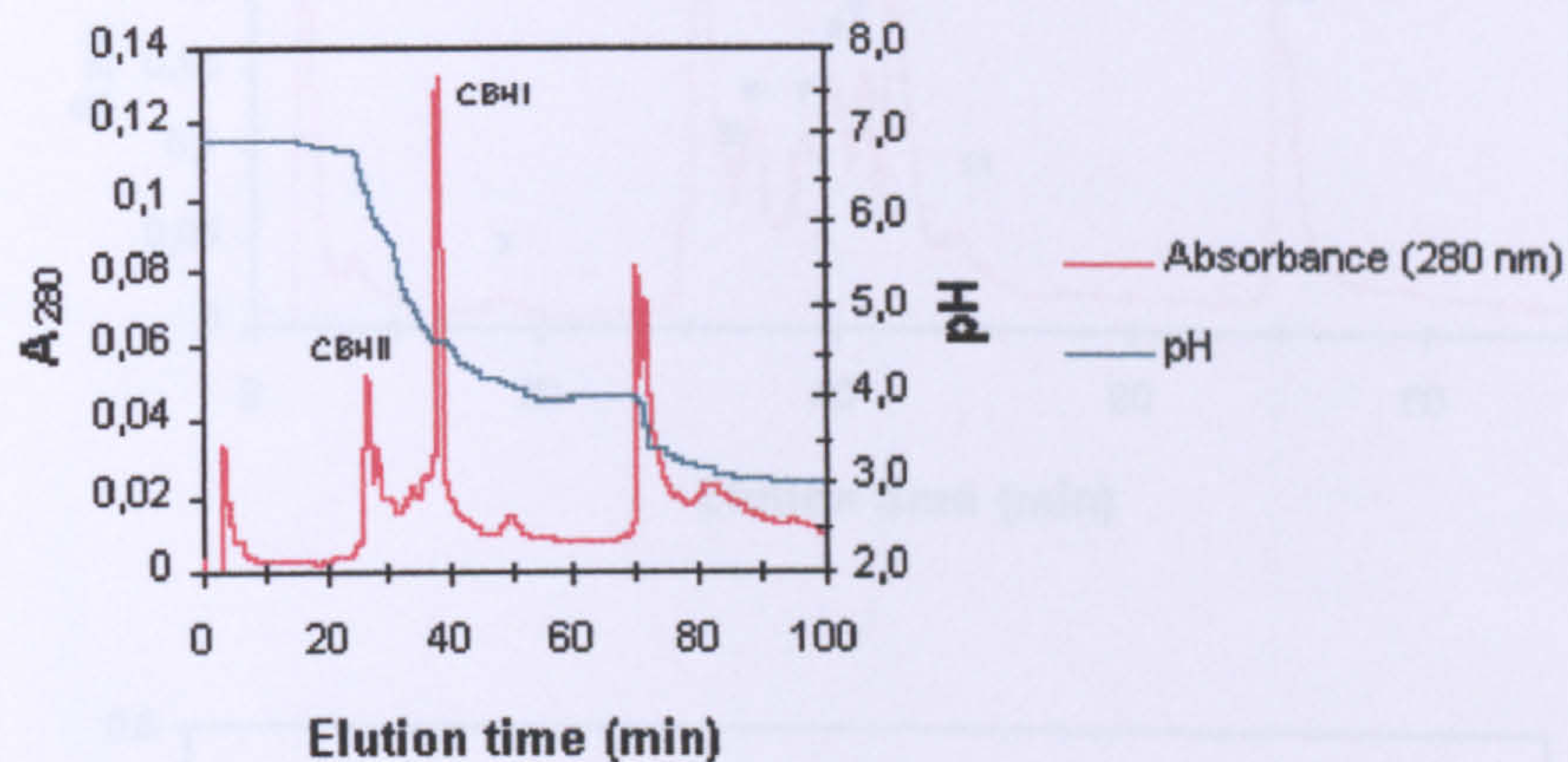
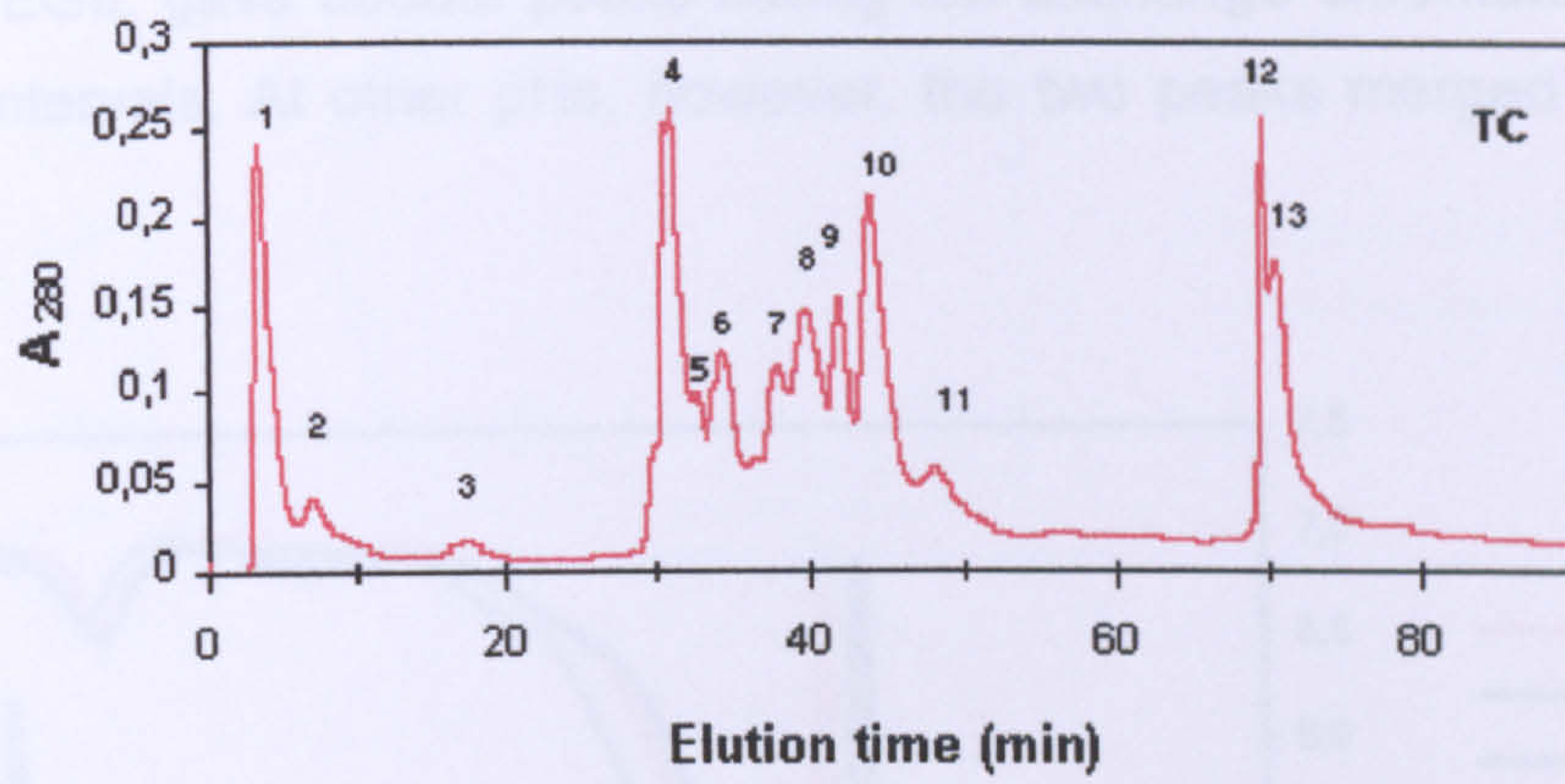
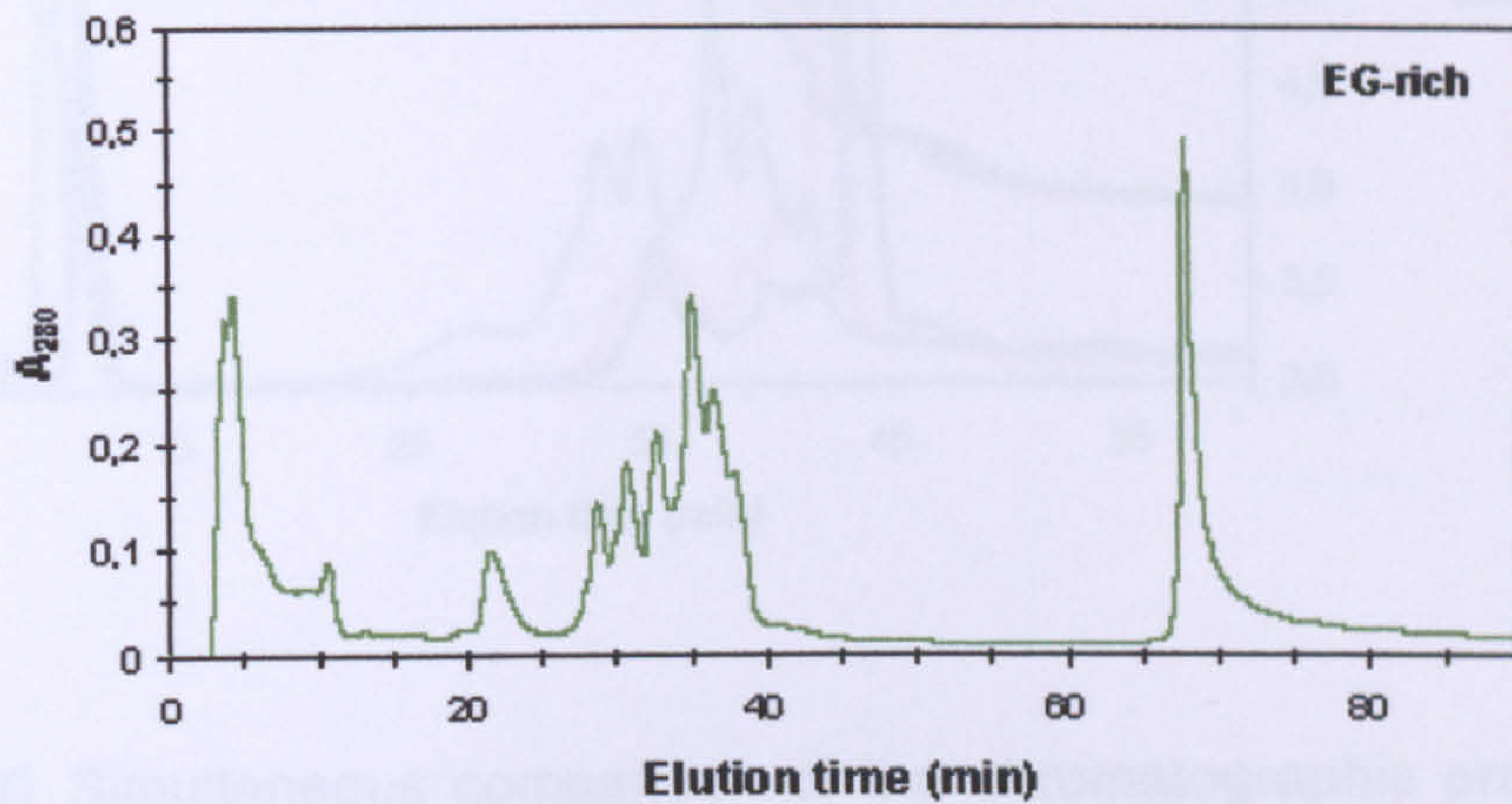


Figure 4.18 Chromatographic profile of CBH-rich preparation (0.1 mg of protein injected).

Since the peaks of the major cellulases are eluted at between 15 and 50 minutes all of this part of the chromatograms will be considered.

In the pH-chromatographic profile (absorbance plotted against pH rather against time) shown in Figure 4.21 it is possible to observe that in the EG-rich preparation the peaks corresponding to the EGs are more pronounced than in TC. In the former preparation the EGs were over produced and their composition in the mixture was 50:50 (see Table 3.1, section 3.2.3). In the CBH-rich profile it can be seen that the peak corresponding to CBHII (peak on the right of the graph, pH 6.0-6.3) is slightly bigger, because in this preparation the production of CBHII was slightly increased (see Table 3.1, section 3.2.3). The EG-rich chromatogram (Figure 4.21) shows four major peaks, two between pH 4.75 and 5.3 and another two between pH 5.5 and 6.3. The first interval may correspond to EGI and the second to EGII. The existence of two peaks close together may be due to different cellulase conformations (isoforms). It has been reported (Medve *et al.*, 1998*b*) that CBHI can be separated in several isoforms and that these have different pIs although their catalytic and adsorption properties are similar. It was also observed that purified preparations of CBHI,





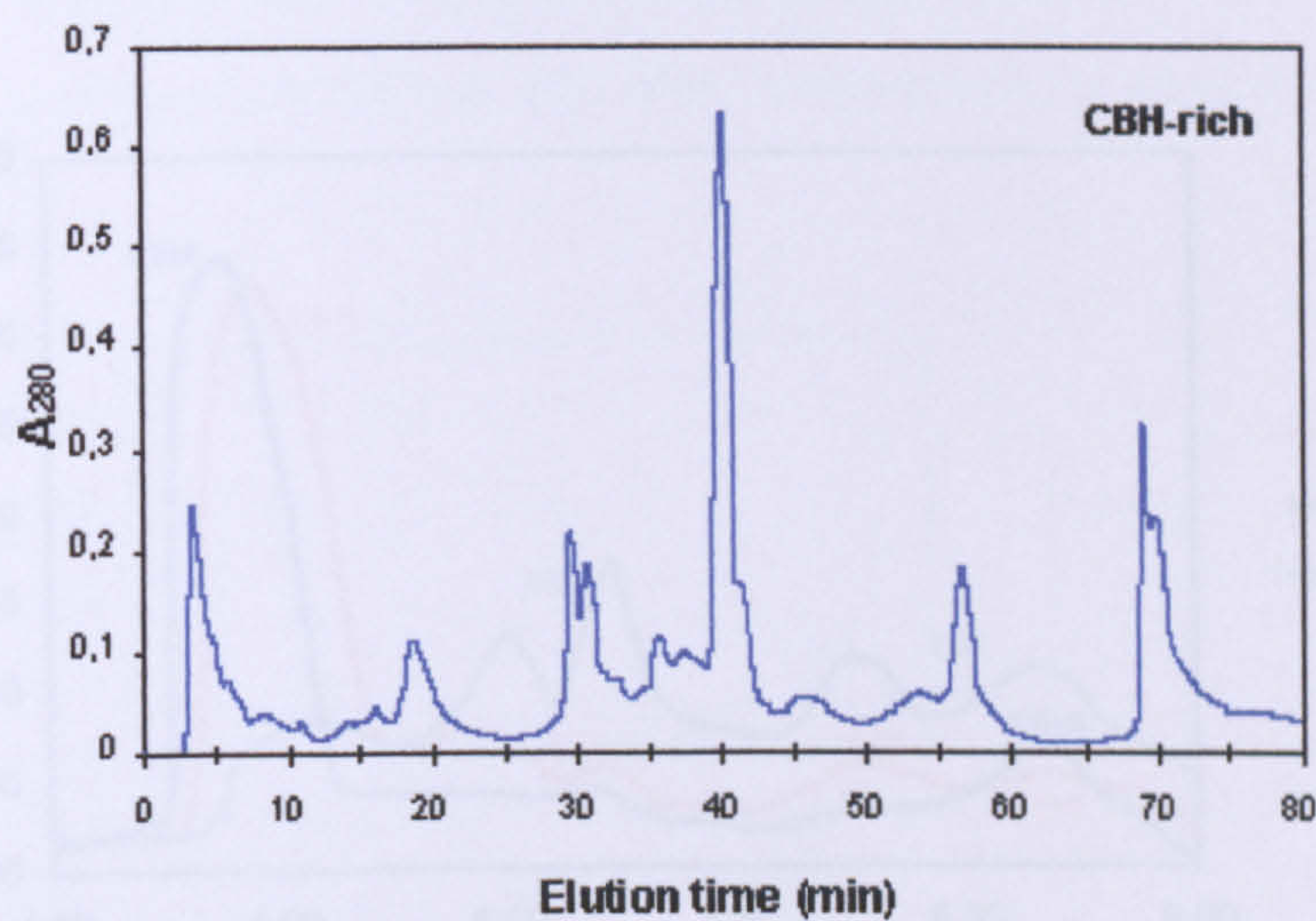


Figure 4.19 Chromatographic profiles of the three cellulase preparations (1 mg of protein loaded).

and also of EGII, gave double peaks during ion-exchange chromatography at certain pH intervals. At other pHs, however, the two peaks merged into each other.

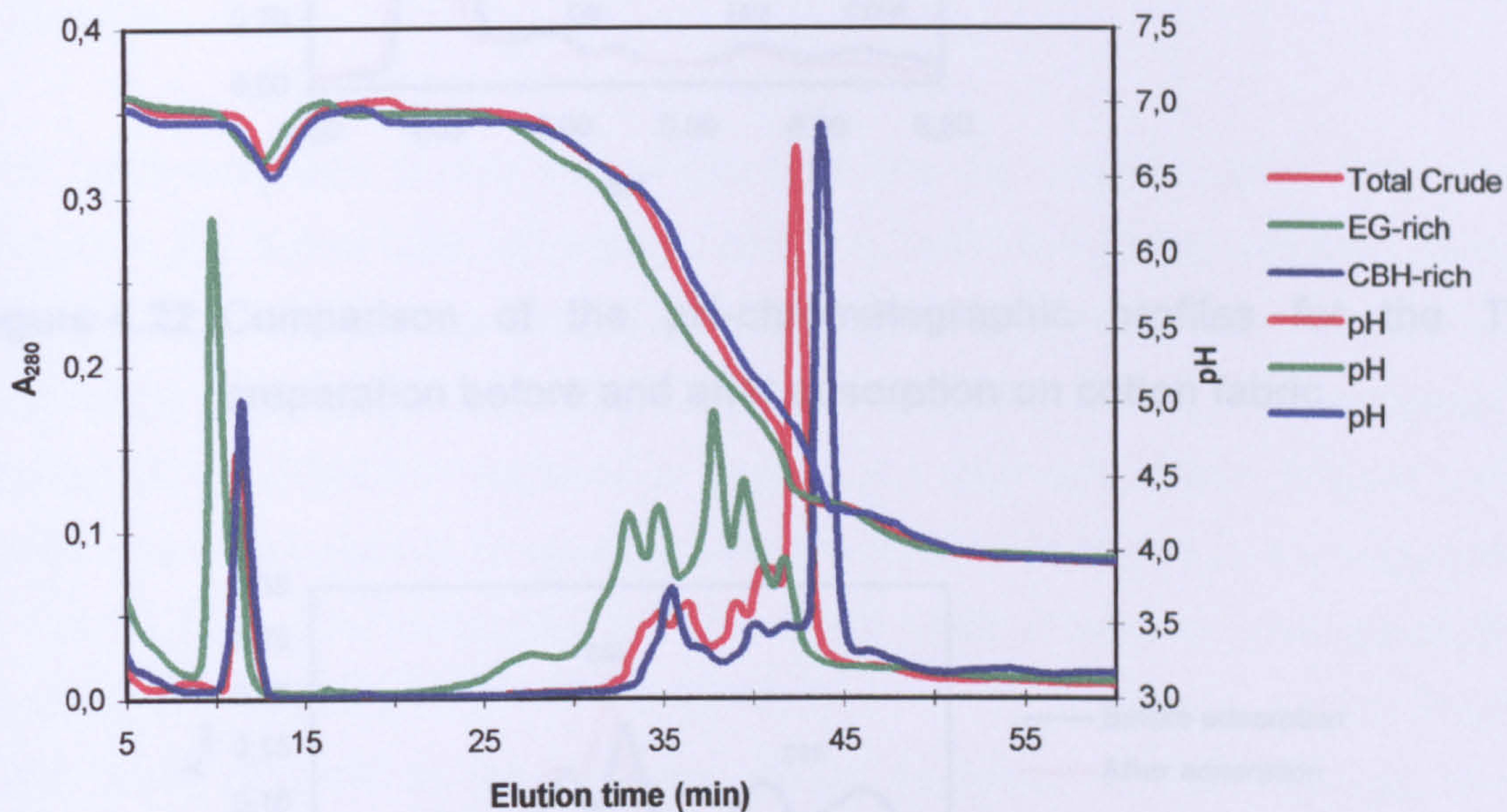


Figure 4.20 Simultaneous comparison of the chromatographic profiles of the three cellulase preparations.

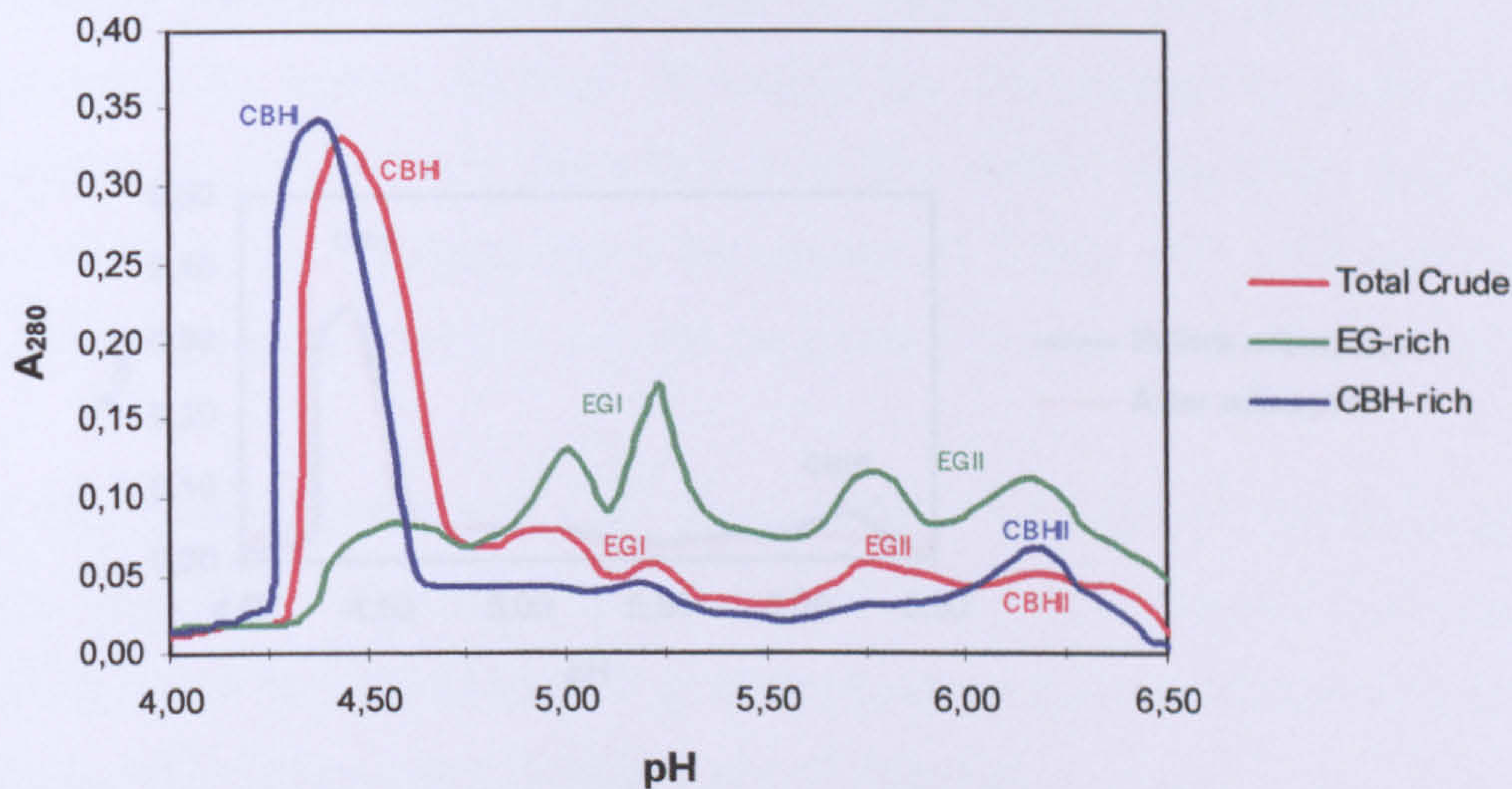


Figure 4.21 pH profile of the three cellulase preparations.

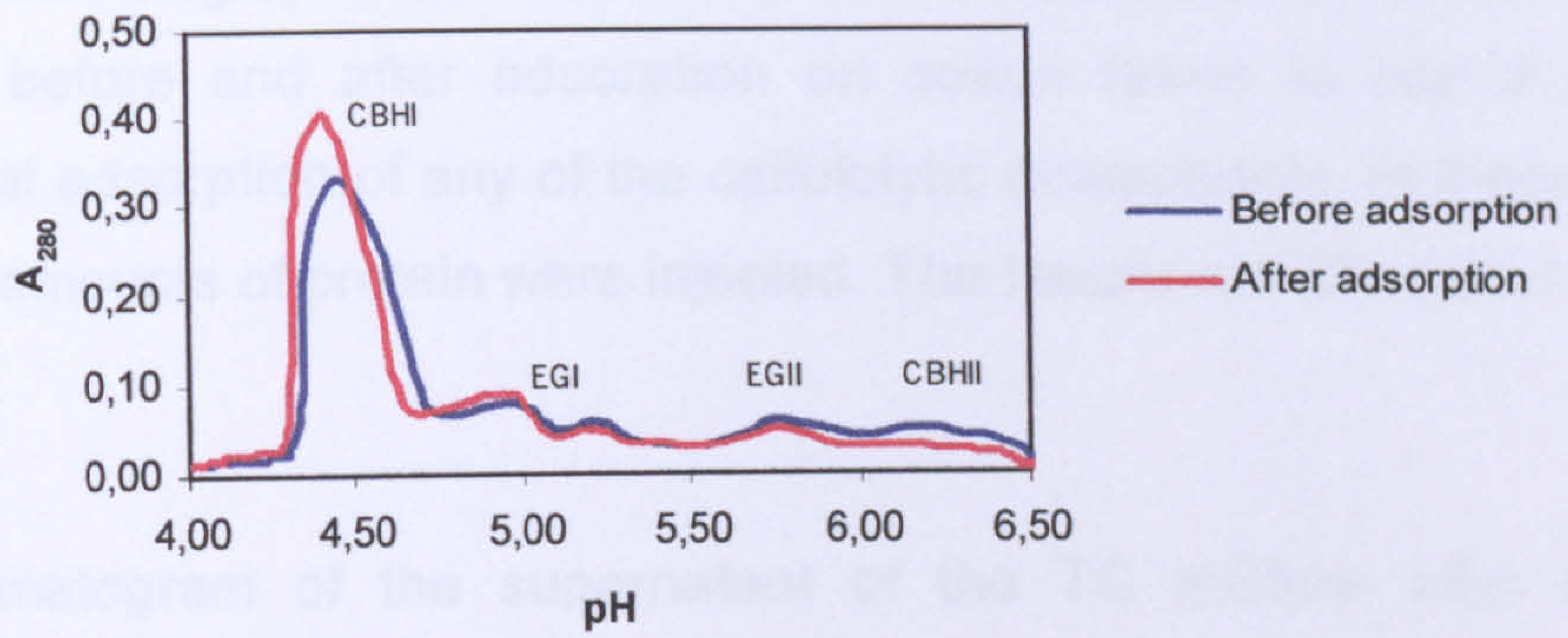


Figure 4.22 Comparison of the pH-chromatographic profiles for the TC preparation before and after adsorption on cotton fabric.

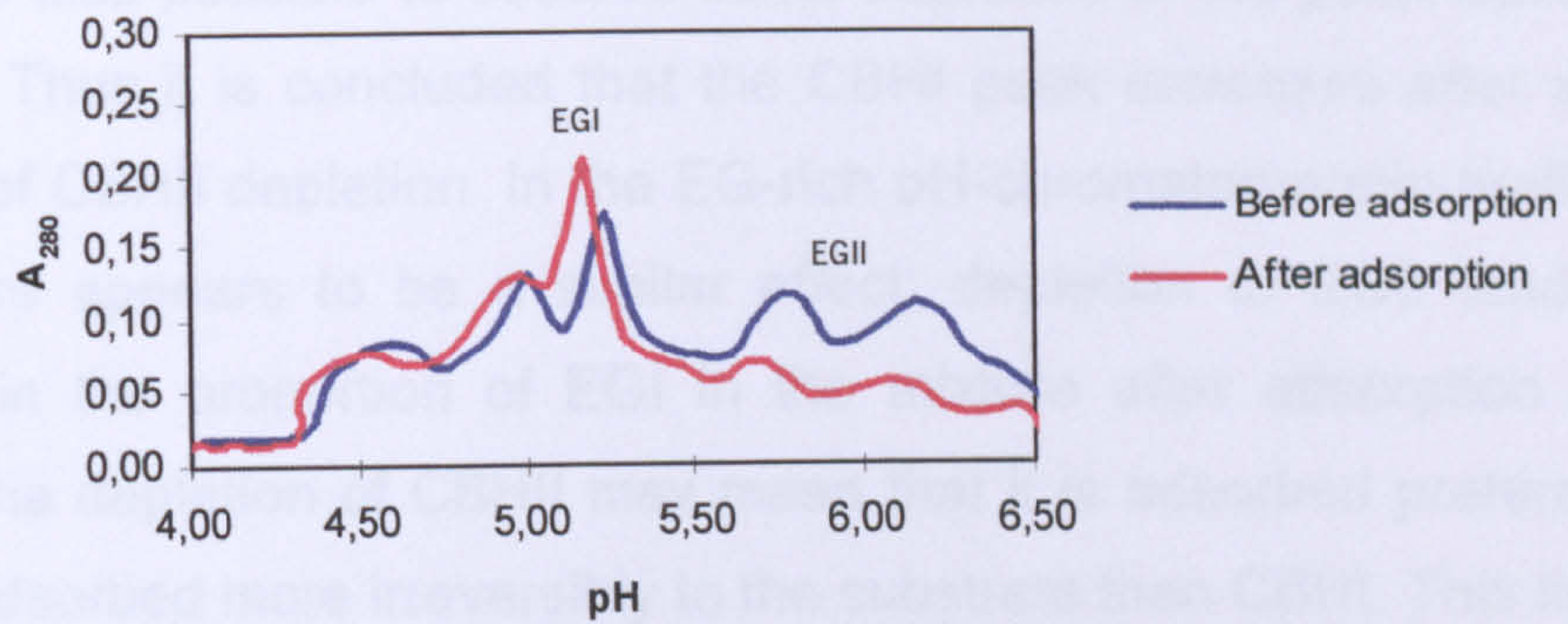


Figure 4.23 Comparison of the pH-chromatographic profiles for the EG-rich preparation before and after adsorption on cotton fabric.

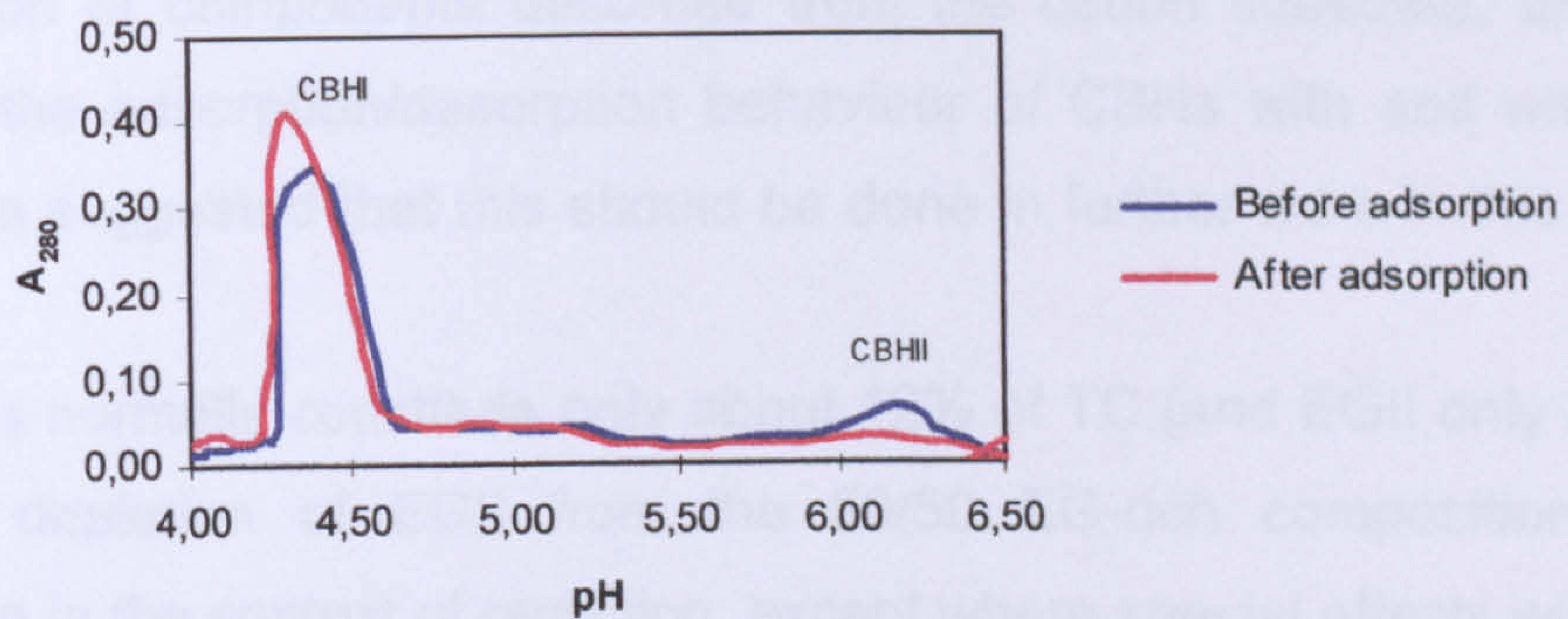


Figure 4.24 Comparison of the pH-chromatographic profiles for the CBH-rich preparation before and after adsorption on cotton fabric.

The pH-chromatographic profiles of the three cellulase preparations were also analysed before and after adsorption on cotton fabric to see if there was preferential adsorption of any of the cellulolytic components. In these analyses the same amounts of protein were injected. The results are illustrated in Figures 4.22-4.24.

The chromatogram of the supernatant of the TC mixture after adsorption (Figure 4.22) shows little difference compared with that of the original mixture, with the exception that the peak corresponding to CBHI seems to increase after adsorption. This result is confirmed by the analysis of the chromatographic profiles of the CBH-rich preparation before and after adsorption (Figure 4.24), where it is also possible to observe some depletion of the peak corresponding to CBHII. Thus it is concluded that the CBHI peak increases after adsorption because of CBHII depletion. In the EG-rich pH-chromatographic profile (Figure 4.23) there appears to be a similar effect; depletion of EGII leading to an increase in the proportion of EGI in the mixture after adsorption on cotton fabrics. The depletion of CBHII may mean that it is adsorbed preferentially, or that it is adsorbed more irreversibly to the substrate than CBHI. This finding is in agreement with the results reported by Linder and Teeri (1996) and by Carrard and Linder (1999), who showed that the adsorption of CBD of CBHI was completely reversible, whereas the CBD of CBHII could not be desorbed from the substrate by buffer dilution. It would be interesting to determine the composition of components desorbed from the cotton substrate, and also to compare the adsorption/desorption behaviour of CBHs with and without their CBDs. It is suggested that this should be done in further work in this area (see 5.2).

Since EGs normally constitute only about 12% of TC (and EGII only about 1 to 2%) the depletion of EGII from the 50/50 EG-rich composition may be misleading in the context of recycling, except where special effects with EG-rich (and particularly EGII-rich) cellulases are of interest.

In the context of recycling TC, these results suggest how the recycled cellulolytic mixture should be adjusted to achieve the levels of synergism

necessary for the hydrolysis of cellulosic substrates. In the case of textile processing, a constant composition of components is extremely important because the finishing effects on cotton substrates depend on subtle changes in fibre surfaces. It would clearly be advantageous to use cellulase compositions in which all components were reversible, so that no adjustments would be necessary. This possibility is discussed further in 4.4.2 and 5.2.

4.4.2 Adsorption, desorption and activities of purified endoglucanases having CBDs from family I and family II

This section of the work is not directly related to the problems of recycling celluases after cotton processing, but the availability of two purified, single component EGs, as entire enzymes and their isolated catalytic domains, provided a unique opportunity to compare the adsorption and activity characteristics, under cotton processing conditions, of EGs having cores and CBDs from different families. The EGs used were:

(i) EGV from *Humicola insolens* and its isolated catalytic domain. This EG (supplied by Novo Nordisk) has a family I CBD and a family 45 catalytic domain;

(ii) CenA from *Cellulomonas fimi* and its isolated catalytic domain. This EG (supplied by Prof. Douglas Kilburn of the University of British Columbia) has a family II CBD and a family 6 catalytic domain.

The results reported here were presented at a recent conference on enzyme applications in textile processing (May 2000, Póvoa do Varzim, Portugal) and have been published in Enzyme and Microbial Technology (Azevedo *et al.*, 2000).

Adsorption/desorption studies

For these studies, 5 g of fabric was incubated with each enzyme at two agitation levels in 50 mL of phosphate buffer (0.1 *M*, pH 7.0) at 50 ºC. The protein dosage used was 1.5 mg protein/g fabric. In low mechanical agitation experiments, the incubation time was 24 hours in flasks held in a shaker bath operating at 125 strokes min^{-1}. The high level of agitation was achieved by adding five stainless steel discs (each disc with average weight of 19.1 g, 32 mm×3mm) to the reaction mixture contained in 500 mL stainless steel pots of a Rotawash machine, rotating at 40 rpm. The incubation time was one hour.

After the first incubation period (1 hour and 24 hours for the high and low agitation, respectively), the protein in solution was measured (see 3.2.4) to calculate the percentage adsorption. The treatment liquor was then diluted (1:2) with phosphate buffer and the mixture was incubated for a further 30 minutes in the same agitation conditions. The concentration of protein in solution was remeasured (see 3.2.4).

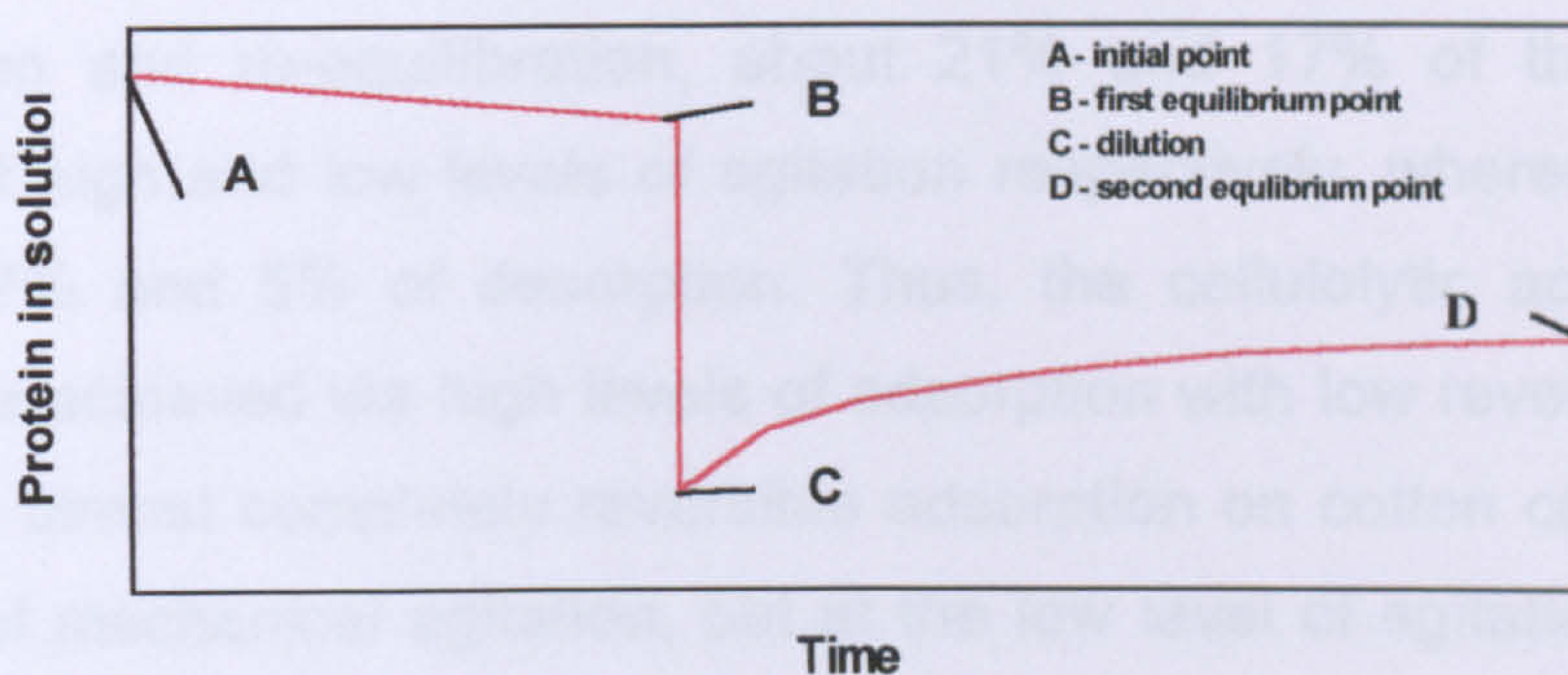


Figure 4.25 Typical adsorption/desorption curve of a protein which binds reversibly.

A typical adsorption/desorption process is represented in Figure 4.25. This shows the free protein concentration returning to a new equilibrium after it has been disturbed by dilution with buffer, demonstrating that the binding of the

protein is reversible. The percentage of adsorption and desorption was calculated as follows:

$$\% \text{ adsorption} = \frac{A-B}{A} \times 100$$

$$\% \text{ desorption} = \frac{D-C}{A} \times 100$$

The results of the adsorption/desorption studies, given in Table 4.8, show that the adsorption of EGV and EGV core was very low for both levels of agitation. This suggests that the action of this endoglucanase (with or without its family I CBD) may be achieved via rapid adsorption/desorption and that its binding to cotton cellulose is highly reversible. This tends to be confirmed by the fact that, after dilution, all protein was desorbed from the fabric, despite the low levels or absence of adsorption after the first period of incubation.

For the CenA enzyme, much higher percentages of adsorption were observed at both agitation levels, 78% and 47% for high and low levels of agitation, respectively. Without the CBD, CenA core showed lower adsorption levels, 27% and 45%, respectively at high and low levels of agitation.

After dilution and re-equilibration, about 21% and 17% of the CenA was desorbed at high and low levels of agitation respectively, whereas CenA core presents 27% and 5% of desorption. Thus, the cellulolytic action of CenA seems to be achieved via high levels of adsorption with low reversibility. CenA core shows almost completely reversible adsorption on cotton cellulose at the high level of mechanical agitation, but at the low level of agitation, only a low degree of desorption was achieved. These results show that the cores are removed from the substrate by high mechanical agitation, whereas entire enzymes (with family II CBD) remain adsorbed even at high levels of agitation. Gilkes *et al.* (1992) and Jervis *et al.* (1997) have reported that CenA enzyme binds rapidly and tightly to cellulose and no net desorption of the protein was observed. Cavaco-Paulo *et al.* (1999), however, have shown, in desorption experiments with isolated CBD of CenA (family II CBD) on cotton fabrics, that at

low mechanical agitation, little desorption occurred, but at higher agitation and also at higher pHs, more protein was desorbed.

Table 4.8 Percentage of protein (± maximum error in protein determination) adsorbed and desorbed after dilution with buffer (1:2) for the four proteins, using high level of mechanical agitation (Rotawash machine, 5 discs, 40 rpm, 50 °C) and low level of mechanical agitation (Shaker bath, 125 rpm, 50 °C).

Protein		Rotawash	Shaker
EGV	Adsorbed	2 ± 4	0
	Desorbed	2 ± 1	-
EGV core	Adsorbed	2 ± 4	0
	Desorbed	2 ± 1	-
CenA	Adsorbed	78 ± 4	47 ± 4
	Desorbed	21 ± 1	17 ± 1
CenA core	Adsorbed	27 ± 4	45 ± 4
	Desorbed	27 ± 1	5 ± 1

Enzymatic treatments

Fabric samples (3.5 g) were incubated with each enzyme in phosphate buffer (0.1 *M*, pH 7.0) at 50 °C, using a fabric to liquor ratio of 1:10. The enzyme dosage used was 1.5 mg of protein/g of fabric in all experiments. Low and high mechanical agitation experiments were carried out as described for adsorption/ desorption studies. For both agitation levels, control tests (identical treatments but without enzymes) were made in order to discount any weight loss caused by agitation in buffer only.

At the end of the treatments, the fabrics were removed from the liquor and treated as described in 3.2.10.

Two independent experiments were carried out for each treatment and the full results are given in Table V in Appendix A. The mean values were used to illustrate the results in Figures 4.26 and 4.27. Enzymatic activity was measured in terms of total fabric weight loss (see 3.2.6). Insoluble weight loss was measured using the method described in section 3.2.7. Soluble weight loss was determined by subtracting the value of insoluble weight loss from the total weight loss. The reducing power, or end group content of fabric samples after enzymatic treatment (for the low level of mechanical agitation experiments) was determined by the method described in 3.2.2.

Results and discussion of enzymatic treatments

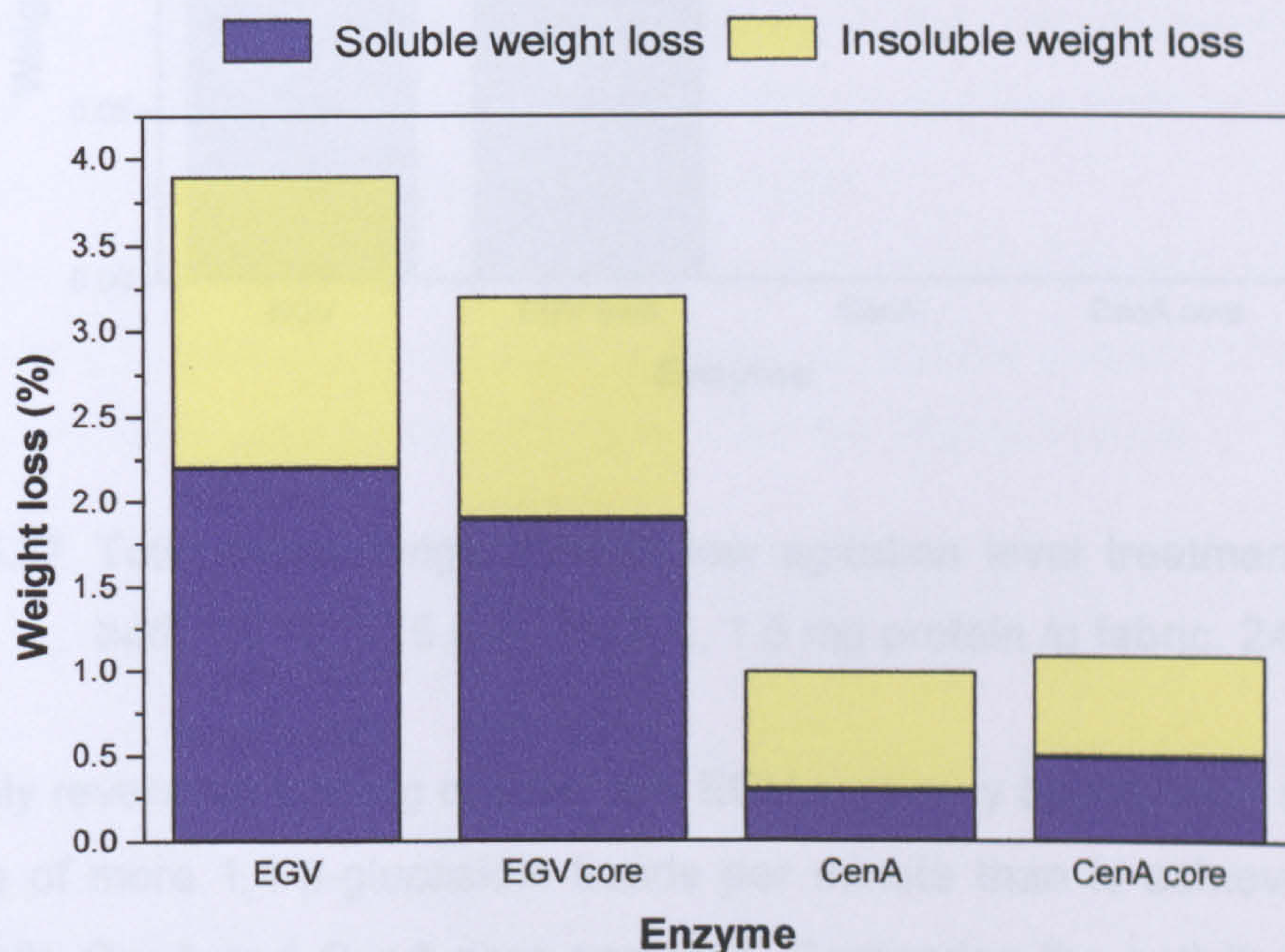


Figure 4.26 Total (soluble and insoluble) fabric weight loss in high agitation level treatment (Rotawash machine, 50 °C, 40 rpm, 5 discs, pH 7.0, 1.5 mg protein/g fabric, 1 h).

From Figure 4.26 it can be seen that EGV and EGV core achieve three to four times the fabric weight loss produced by CenA and CenA core under the same high agitation conditions. Although endoglucanases from different sources are

expected to have different specific activities, it is reasonable to suggest that these differences could result mainly from their different adsorption/desorption characteristics. Jervis *et al.* (1997) have also reported that CenA has only moderate activity on crystalline cellulose.

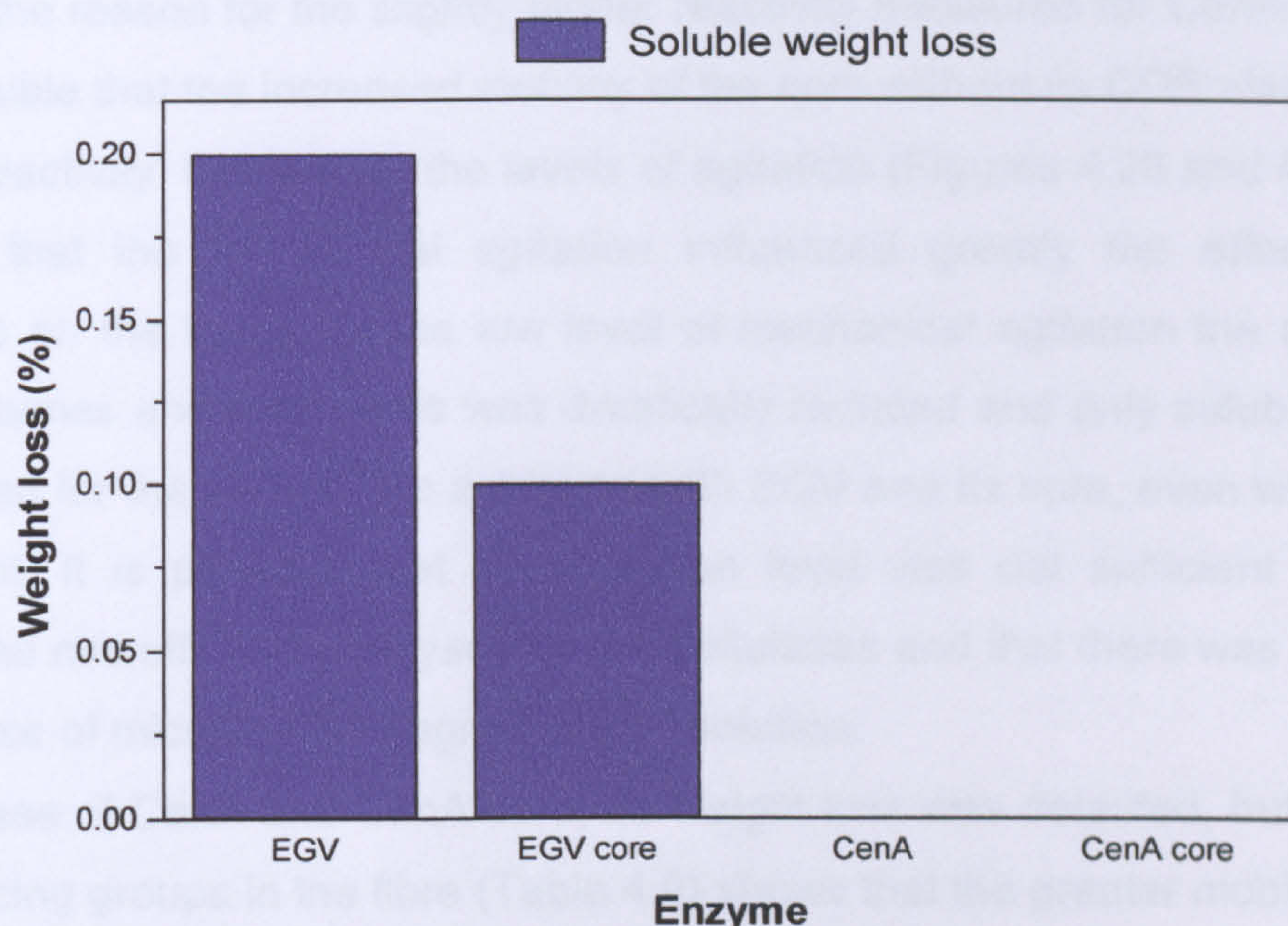


Figure 4.27 Total fabric weight loss in low agitation level treatment (Shaker bath, 50 ºC, 125 rpm, pH 7.0, 1.5 mg protein /g fabric, 24 h).

The highly reversible binding of EGV and EGV core may be the main reason for cleavage of more 1,4-β-glucosidic bonds per minute than is achieved by the less mobile CenA and CenA core enzymes. Comparing the activity results of EGV and EGV core (Figures 4.26 and 4.27), it is possible to notice that at the high level of agitation the enzymes show similar activities. At the low level of agitation, the soluble weight loss produced by EGV core is about half of that obtained by the intact enzyme. These results suggest that the presence of CBD is not essential for the hydrolysis of cotton cellulose (even at low agitation) by the EGV of *H. insolens*.

The hydrolysis experiments made with CenA endoglucanase showed no significant differences between the activities of CenA and its core. It should be noted that equal amounts of proteins were used in these experiments and there was therefore a slightly higher molar concentration of CenA core present, compared with the catalytic domain concentration for the entire enzyme. This may be the reason for the slightly higher reactivity measured for CenA core, but it is possible that the increased mobility of the core without its CDB also leads to higher reactivity. Comparing the levels of agitation (Figures 4.26 and 4.27), it is evident that the mechanical agitation influenced greatly the effect of the enzymes on the fibres. At the low level of mechanical agitation the activity of both enzymes and their cores was drastically reduced and only soluble sugars accounted for the weight loss achieved with EGV and its core, even with longer treatment. It is possible that the agitation level was not sufficient to break weakened microfibrils hydrolysed by the cellulases and that there was therefore no release of microfibrillar fragments into solution.

In the case of CenA and CenA core, no weight loss was detected, but analysis for reducing groups in the fibre (Table 4.9) shows that the greater mobility of the core again leads to slightly higher activity than the whole enzyme.

Table 4.9 Reducing groups produced on the fibre by the CenA proteins at low agitation (Shaker bath, 50 °C, 125 rpm, pH 7.0, 1.5 mg protein/g fabric, 24 h). Values in brackets represent the standard deviation of two independent experiments.

Fabric Treatment	Reducing groups (mg glucose/ g cellulose)
CenA	0.29 (0.01)
CenA core	0.34 (0.02)
Buffer only	0.12 (0.01)

From the point of view of cellulase recycling it would clearly be an advantage to use cellulase compositions in which all components exhibit highly reversible

adsorption (like EGV from *H. insolens*). There would then be no need to "top up" with depleted components and the potential cost savings would be increased. Other workers (Linder and Teeri, 1996) have shown that CBHI adsorption is highly reversible and it seems likely that synergistic compositions of EGs and CBHs could be designed (see 5.2).

4.4.3 Effects of surfactants on enzymatic treatments and desorption of cellulases from the substrate

Interactions between surfactants, cellulose and cellulases used in the hydrolysis of cotton fabrics are important for two reasons. Firstly, the presence of surfactants in the treatment bath lowers the interfacial tension and thereby improves the uniformity of wetting and penetration of liquor into the fabric. This is believed to improve the accessibility, of cotton fibre surfaces within the fabric structure, to cellulase adsorption and may enhance cellulase activity. On the other hand, depending on the ionic nature of the surfactant, interaction between the surfactant and cellulase protein may cause partial deactivation and a reduction in activity.

Secondly, surfactants may assist the desorption of cellulase from cellulose surfaces and this may be of importance for improving the efficiency of cellulase recycling.

In this work the activities of total cellulases from different sources (*Trichoderma reesei* and *Humicola insolens*) were investigated in the presence of the anionic and nonionic surfactants described in 3.1.1. The recovery of TC activity (*T. reesei* only) from treated cotton was compared using buffer only and buffer containing anionic or nonionic surfactant as desorbent.

To compare the effects of the anionic and nonionic surfactants on cellulase activity, hydrolysis experiments in the presence and absence of these compounds were performed. The same enzyme concentrations (3.6 mg protein/g fabric) and incubation conditions (Rotawash machine, 50 ºC, 20 rpm, 1 h) were used in buffer only and buffer plus anionic or nonionic surfactant (both used at 1 g/L). In the case of *Humicola insolens* cellulase (neutral cellulase) the activity was determined in phosphate buffer solution (0.1 *M*, pH 7.0). Activities were measured in terms of the soluble reducing sugars produced (see 3.2.1) in each liquor treatment.

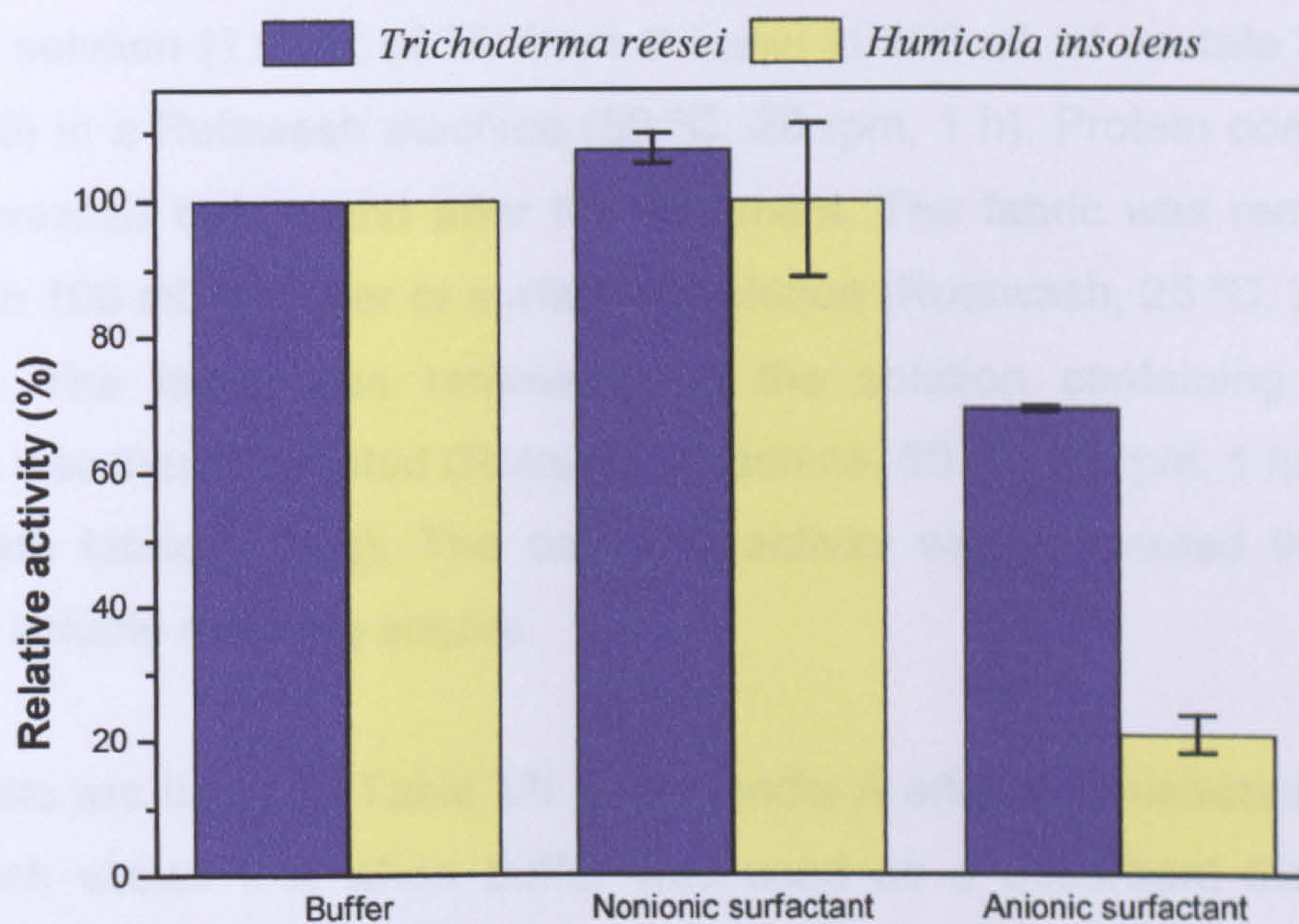


Figure 4.28 Effect of surfactants (1 g/L) on cellulase activity during enzymatic hydrolysis of cotton fabrics (Rotawash machine, 3.6 mg protein/g fabric, 50 ºC, 20 rpm, 1 h). The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

The results of these experiments are given in Table VI in Appendix A and are illustrated in Figure 4.28 which shows that the nonionic surfactant increased the activity of TC from *T. reesei* by about 7% but the effect on *H. insolens* cellulase was less consistent. The anionic surfactant, which is known to cause partial denaturation of cellulases (Otzen *et al.*, 1999) caused some deactivation of both cellulases – the *T. reesei* cellulase retained about 70% activity whereas *H. insolens* cellulase retained only about 20% of its original activity.

Desorption of cellulases from cotton fabrics using buffer and surfactants

To compare the effectiveness of surfactants and buffer only for the desorption of cellulases from fabric, pieces of cotton fabric (6.9 g) were incubated in a cellulase solution (1.0 mL of TC from *T. reesei* in 100 mL of acetate buffer, 0.1 *M*, pH 5.0) in a Rotawash machine (50 °C, 20 rpm, 1 h). Protein concentration was determined before and after the treatment. The fabric was removed and washed in 100 mL of buffer or surfactant solution (Rotawash, 25 °C, 20 rpm, 30 minutes). The fabric was removed and the solution containing desorbed enzymes was then incubated (Rotawash machine, 50 °C, 20 rpm, 1 hour) with a new cotton fabric (6.9 g). The cellulase activity was measured in terms of liberated soluble reducing sugars.

The results are listed in Table VII in Appendix A and are illustrated in Figure 4.29 which shows that when buffer was used as a desorbent the resulting solution had 31.7% of the original activity towards cotton fabric. Analysis of the liquor after the first hydrolysis showed that 30-35% of the enzyme protein was adsorbed, but it must be remembered that the wet fabric also carried free enzyme over into the desorption bath. It is therefore not clear what percentage desorption of protein is achieved in these experiments and there is a scope for more detailed study in this area. There was no evidence that buffer plus surfactants desorbed more protein than buffer alone. Unfortunately the protein concentrations in presence of surfactants could not be measured by the

Bradford (or Lowry) method because of the interference of surfactants with the formation and colour of the dye-protein complex.

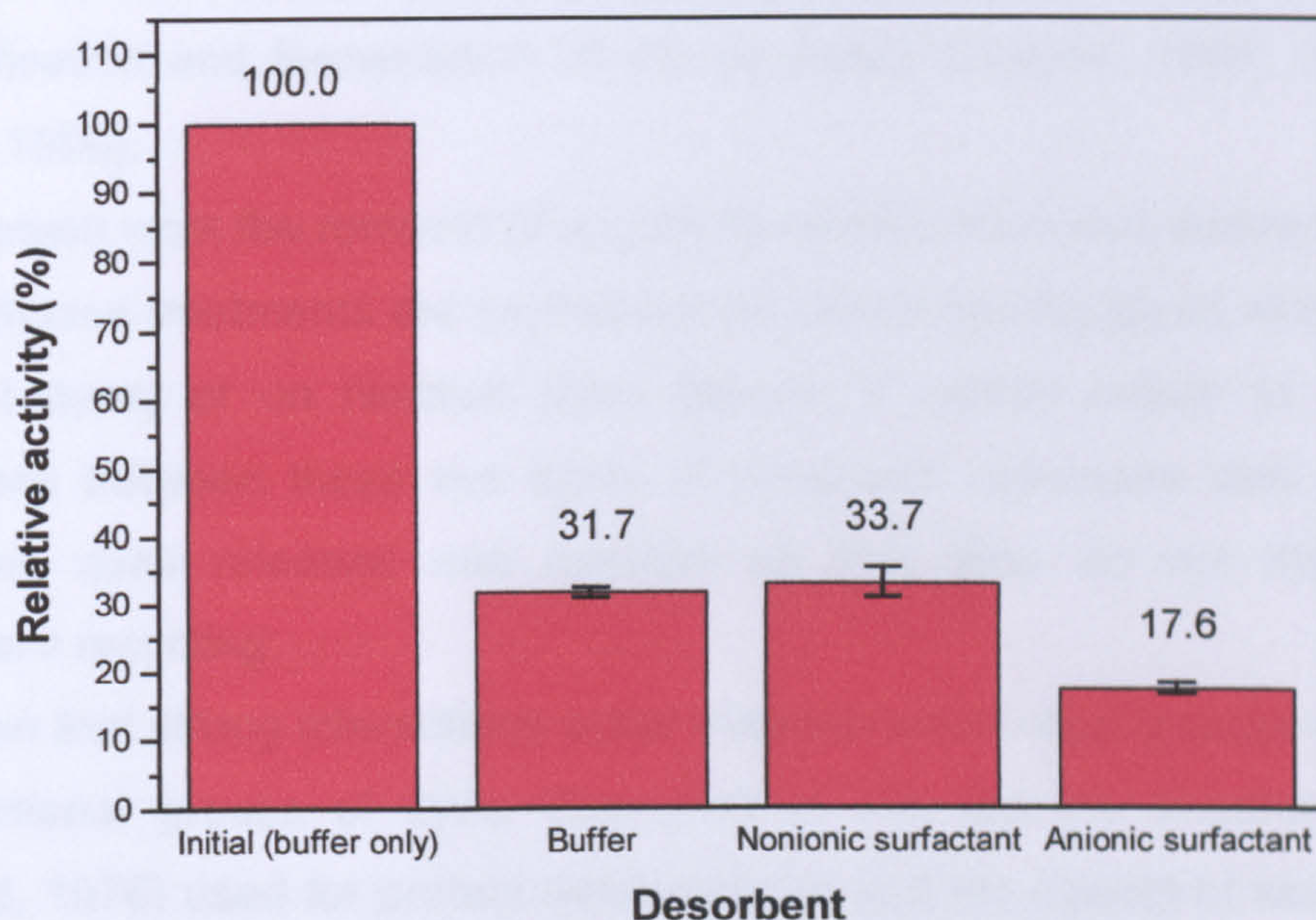


Figure 4.29 Comparison of the desorbed activities (as liberated reducing sugars) using different desorbents. The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

4.5 Concentration of cellulases by ultrafiltration after fabric treatments

4.5.1 Removal of sugars and dyes from processing liquors using an ultrafiltration system

When cellulase solutions are recycled, there is an accumulation of reaction products (mainly cellobiose and glucose), which will eventually cause inhibition (4.2.1). These soluble sugars should therefore be eliminated from the recycled solution when their concentration becomes sufficient to cause a significant reduction in cellulase activity. Several methods have been proposed to achieve

this. These include the elimination of sugars by ultrafiltration (Franz and Blaschek, 1990; Gregg and Saddler, 1996), the supplementation of the cellulolytic system with exogenous β-glucosidase activity, or the simultaneous saccharification and fermentation of the substrate (Dekker, 1986; Gregg and Saddler, 1996).

In the present work the removal of sugars by ultrafiltration was examined. Since many cellulase treatments are carried out on denim fabrics (dyed with indigo or other vat dyes) or on reactive dyed fabrics, it makes sense to study the interactions between these two types of dyes and cellulases with a view to eliminating dyes released into solution so that they do not interfere on subsequent recycling.

It is known that strong interactions occur between amino acid groups of proteins and functional groups of dyes. Examples of this are the Bradford method (Bradford, 1976) used for protein determination and the dyeing of wool (protein fibre). It was, therefore, particularly important to study the interactions between cellulase enzymes and indigo and the reactive dyestuff (Remazol Brilliant Blue R) used in the present study. Clearly, when recycling of enzymes is intended, it is desirable to have no dyes present in the recycled solution, because if these are not removed they may cause backstaining.

Two dyes, a reactive – Remazol Brilliant Blue R and a vat dye – indigo, were chosen to study their influence on the hydrolysis of cotton fabrics and their subsequent separation from the reaction mixture by ultrafiltration during enzyme recovery. The separation was also carried out at different pHs in order to investigate the interaction between the dyes and the enzymes.

Enzymatic treatments and preparation of treatment liquors for ultrafiltration

Dyed or undyed fabric samples (25 g) were placed in a pot of a Rotawash machine with 250 mL of the cellulase solution in acetate buffer (0.1 *M*, pH 5.0). An enzyme load of about 100 mg protein/g of fabric was used with sodium

azide (0.02%) being added as an antimicrobial agent. The incubation was performed at 50 °C, 20 rpm during 8 hours. To increase the level of mechanical agitation, 10 stainless steel discs were added to the reaction mixture. Control treatments were made with the fabrics in buffer only under the same incubation conditions. Two replicates were done for each fabric treatment.

After the enzymatic treatments, the solutions were filtered through a 2.7 μm glass microfibre filter GF/C (Whatman, Maidstone, England). The retained solids on the filters and the treated fabrics were washed with acetate buffer (1 L) to elute adsorbed enzymes. Samples of the filtrates and washings were collected for analysis of protein, soluble reducing sugars and dye in the case of fabric dyed with Remazol Brilliant Blue R. The two portions were mixed and buffer was added to make a volume of 2 L for the ultrafiltration experiments.

For studies of dye-protein interactions at different pHs, the same procedures were followed but using 5 g of fabric dyed with Remazol Brilliant Blue R or indigo in 50 mL of cellulase solution. In this case agitation was provided by two stainless steel discs. After filtration, washing was with 100 mL of buffer and the final volume was made up to 250 mL with buffer.

Ultrafiltration experiments

The solutions (2 L) obtained from the procedure described above were ultrafiltered using a Prep/Scale™ TFF cartridge (Millipore Corporation, Bedford, MA, USA), containing a 0.09 m^2 polysulphone ultrafiltration membrane with a MWCO of 10 kDa. Before ultrafiltration of the hydrolysate solutions, the ultrafilter was pre-washed with acetate buffer. The ultrafiltration was performed at 1-1.2 bar until the desired reduction in volume of the retentate was achieved (see Figure 4.30). Samples of the feed, retentate and permeate solutions were analysed for protein (see 3.2.4), sugars (see 3.2.1) and dye content (see 3.3.8). After ultrafiltration, the cartridge was washed with buffer to recover protein present in the solution that remained inside the ultrafilter and in the lines. Ultrafiltration of a sugar solution (containing glucose and cellobiose dissolved in

acetate buffer) was also made to study the remotion of sugars in the absence of proteins.

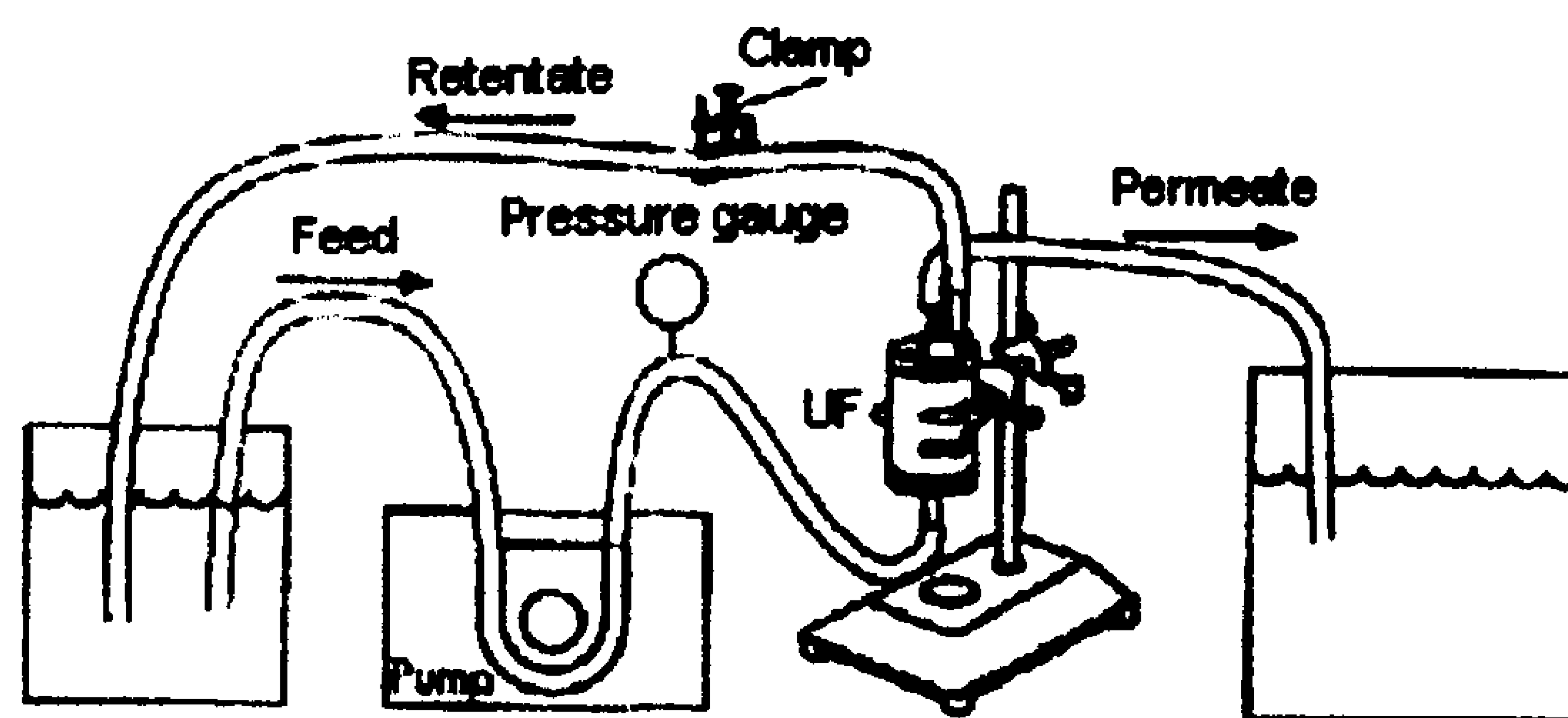


Figure 4.30 Schematic representation of the system used for ultrafiltration experiments.

To study the influence of pH on dye separation from the reaction mixture, ultrafiltration was carried out on a smaller scale. Portions (20 mL) of feed solution were separated for pH adjustment in a range from pH 3 to 9, using sodium hydroxide or acetic acid. These experiments were made using a 4 mL centrifugal ultrafiltration unit with polysulphone membranes having a 5000 Da MWCO (Ultrafree-4, Millipore Corporation, Bedford, MA, USA). The ultracentrifugation was carried out at room temperature in a centrifuge tube EBA 8 (Hettich, Tuttlingen, Germany) (2875 *g*) with 4 mL of feed solution during 1 hour. This procedure was repeated three times and the retentate and permeate were taken for analysis of protein, sugars and dye. The ultrafilters were washed with 4 mL of acetate buffer.

The results in Table 4.10 show the levels of cellulase adsorption and the sugar production for undyed and dyed fabrics. There were no significant differences in the adsorption percentage for dyed and undyed fabrics, although adsorption was less reproducible on the dyed fabrics. With respect to the liberation of reducing sugars, it seems that the presence of a reactive dye inhibited the hydrolysis rate.

Table 4.10 Protein (TC) adsorption (on the fabric plus fine cotton particles) and sugar production after the enzymatic hydrolysis using undyed and dyed cotton fabrics as substrates (Rotawash machine, 50 °C, pH 5.0, 100 mg protein/g fabric, 20 rpm, 10 discs, 8 h). Values in brackets represent the standard deviation of two independent experiments.

Fabric	Protein adsorption (%)	Sugar production (g)
Undyed	37.1 (1.1)	0.709 (0.093)
Dyed with Remazol Brilliant Blue R	39.6 (6.8)	0.451 (0.201)
Dyed with indigo	38.2 (6.6)	1.215

This is in accordance with the findings of other authors (Buschle-Diller and Traore, 1998; Cavaco-Paulo *et al.*, 1998; Choe *et al.*, 1995; Koo *et al.*, 1994). It was also shown that the presence of indigo does not inhibit the cellulase activity, and in this experiment (single result only) there was increased activity on the indigo-dyed fabric, but this is not in agreement with previous findings (Cavaco-Paulo *et al.*, 1998; Choe *et a.l*, 1995; Koo *et al.*, 1994).

After fabric treatments the enzymes are distributed between the hydrolysate, particles of cotton debris and the cotton substrate (adsorbed). In principle the cellulases can be recovered from both phases of reaction and recycled for further treatments. The proteins that remained in solution were recovered from the reaction mixture by simple filtration to separate the soluble compounds from the debris. This recovery was incomplete, however, since some enzymes remained adsorbed to the fabric and to the cotton particles (debris). Washing of the fabric and the debris, which was retained on the filter, allowed some further enzyme recovery by simple desorption. Table 4.11 represents the percentage recovery which can be achieved from the filtrate and from the washing process after hydrolysis of undyed and dyed cotton fabrics. It is possible to observe that the greater part of protein remained in the filtrate, but that a significant

percentage was also recovered by washing of the fabric and the debris (15 to 18%). With these processes it was possible to achieve a total cellulase protein recovery of 75 to 80%. The presence of dyes on the substrate made no significant difference to the percentage protein recovery.

The 20-25% loss of protein must be due to incomplete desorption of the cellulases from the fabric and cotton debris. To obtain higher levels of desorption greater volumes of buffer and or different conditions should be used for washing. If more buffer solution is used more dilute enzyme solutions are obtained, but this can be easily overcome by protein concentration using ultrafiltration. A cost balance should therefore be made in order to investigate if it is viable to use more buffer to desorb the enzymes, or to use other potential desorbents in order to get greater levels of desorption with less buffer volume, and thus less need for subsequent protein concentration. After the recovery procedure ultrafiltration was carried out to concentrate the proteins and remove the sugars and dyes.

Table 4.11 Protein recovery after hydrolysis using undyed and dyed fabric as substrates. Values in brackets represent the standard deviation of two independent experiments.

Fabric	Sample	Protein recovery (%)
	Filtrate	62.9 (1.1)
Undyed	Washing Liquid	18.1 (2.6)
Dyed with Remazol	Filtrate	60.4 (6.8)
Brilliant Blue R	Washing Liquid	15.8 (1.2)
	Filtrate	61.9 (4.4)
Dyed with indigo	Washing Liquid	17.3 (0.4)

Table 4.12 Percentage volume, sugar and dye content of permeate solution relative to the initial feed solution.

Sample	Volume (%)	Sugar (%)	Dye (%)
Sugar solution	61.0	56.8	-
(glucose + cellobiose)	82.4	76.7	-
Hydrolysate of undyed fabric	81.0	58.9	-
(soluble sugars + proteins)	80.4	58.5	-
Hydrolysate of dyed fabric with	79.5	60.4	54.1
Remazol Brilliant Blue R (soluble	78.8	52.5	43.0
sugars + soluble dye + proteins)			
Hydrolysate of dyed fabric with indigo			
(soluble sugars + dye + proteins)	77.9	60.4	None

The results (Table 4.12) show that when only soluble sugars were present in the feed solution, it was possible to achieve about 77% of sugar remotion, when the permeate represented about 80% of the initial feed volume. The presence of proteins in the feed solution caused a reduction in remotion of sugars. It seems that there is some interaction between cellulases and their degradation products which prevents the latter from passing through the filtration membrane. Possibly, some sugars remain "adsorbed" or "attached" to the enzymes causing them to remain together in the retentate. It is known that some cellulases have sugars in their composition – glycosylation of the linker (Medve *et al.*, 1998*b*) which may increase their affinity for other sugars. With respect to the protein in the retentate, about 90-95% was found to be present in this solution. Washing of the ultrafilter and analysis of the resultant retentate showed that there was some protein remaining in the system and this usually constituted 5-10% of the initial amount. Thus 95-100% of the protein was accounted for.

When reactive dye was also present in solution, only about 50% of the dye was removed. This is probably due to strong protein-dye interactions, which may also involve protein-sugar interactions, because reactive dyes bind covalently to cellulose, and after cellulolytic hydrolysis, reactive dyes that appear in solution remain bound to glucose, cellobiose or cellulose oligomers (Rendle *et al.*, 1994). When indigo was present none of this dye passed through the filtration membrane. This is not surprising because as a vat dye, indigo is insoluble in water. To be solubilized it has to be reduced to its acid leuco form (yellow). In this form it penetrates the fibres and only after it has been oxidised it becomes blue and insoluble. After the enzymatic hydrolysis of indigo-dyed cotton fabrics, the indigo trapped in the fibres is released into solution in an insoluble form. There is, however, some solubilization of the dye, due to interaction between the dye and the enzymes (Campos *et al.*, 2000). Since indigo cannot be separated (by ultrafiltration) from cellulases after enzymatic stonewashing, and it is known that the indigo-cellulase complex causes backstaining, it seems unlikely that cellulases used in this application can be recycled unless some other means is found for separating indigo from cellulases.

Effect of pH on reactive dye removal

Proteins are amphoteric molecules, which can be positively or negatively charged, depending on solution pH. Since many dyes are also amphoteric it seems probable that the major interactions between proteins and dyes will be electrostatic. To study this kind of interaction, ultrafiltration experiments at different pHs were performed on the hydrolysates of Remazol Brilliant Blue R- and indigo-dyed fabrics. In these experiments the percentage volume, sugar and dye content of the permeate solution, relative to the initial feed solution was determined.

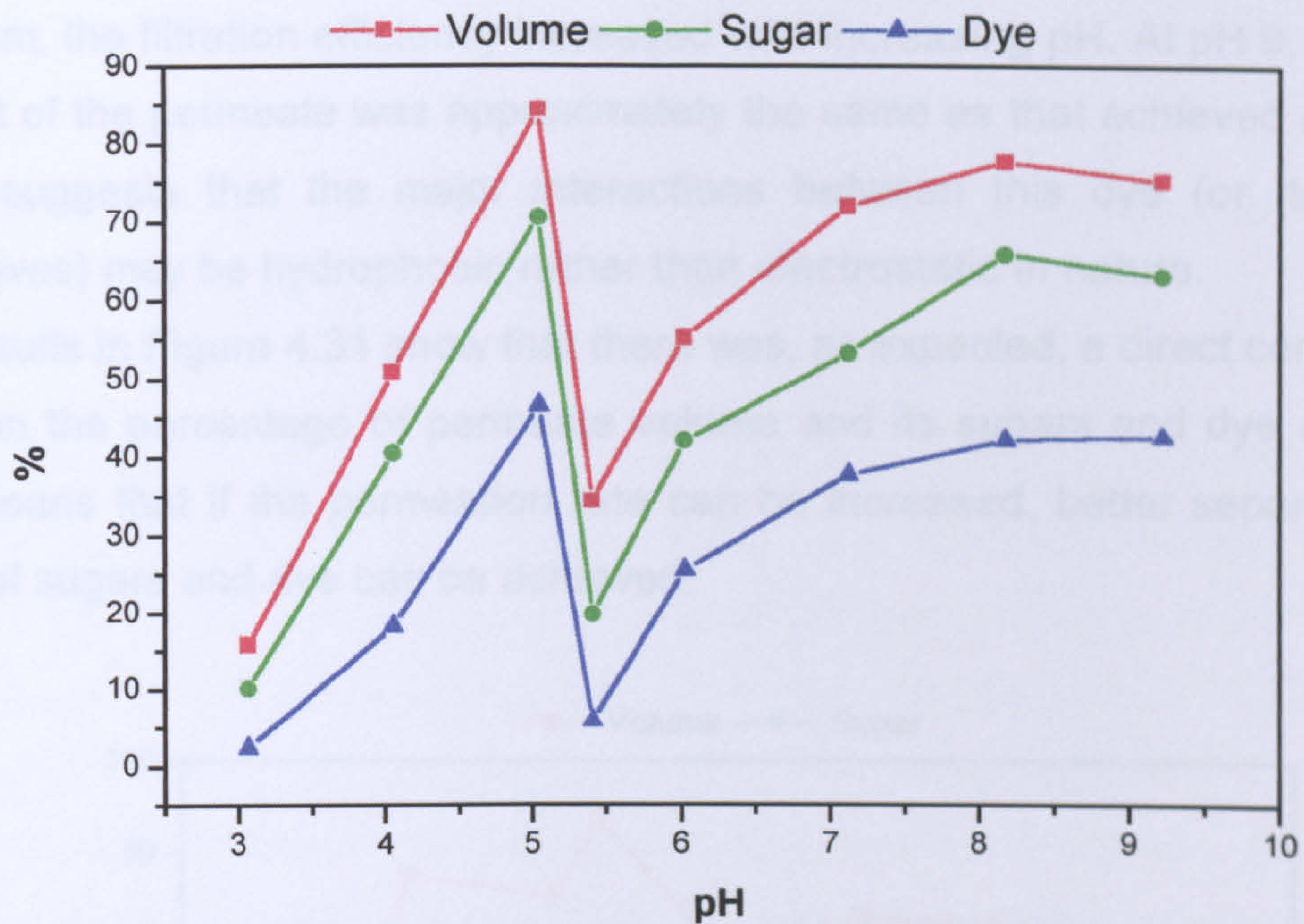


Figure 4.31 Percentages of volume, sugar and dye content of the permeate, relative to the initial feed solution, at different pHs for the hydrolysate of fabric dyed with Remazol Brilliant Blue R.

It can be observed from Figure 4.31, that there is a peak at pH 5 where maximum ultrafiltration efficiency occurs (higher permeate volume and higher separation of sugar and dye). There are also two minima (pH 3 and 5.3) of lower efficiency separation. In the case of pH 3, the enzymes become denatured and deactivated. It was observed that, at this pH, solution presented a turbid aspect and after sedimentation the bottom of the tube showed a deeper blue colour than the top, due to the precipitation of protein together with the dye. Since there was precipitation of the protein in the feed solution, it was more difficult to ultrafilter the solution and hence the low volume of permeate. pH 5.3 corresponds to the hydrolysate resulting from the hydrolysis without any pH adjustment but there is no obvious explanation for the maximum interaction between dye and enzyme protein occurring at this pH. It may be, however, that any addition of electrolyte (to adjust pH) causes some dissociation of the dye-

protein complex leading to greater ultrafiltration efficiency. After this second minimum, the filtration efficiency increased with increasing pH. At pH 9, the dye content of the permeate was approximately the same as that achieved at pH 5, which suggests that the major interactions between this dye (or its sugar derivatives) may be hydrophobic rather than electrostatic in nature.

The results in Figure 4.31 show that there was, as expected, a direct correlation between the percentage of permeate volume and its sugars and dye content. This means that if the permeation rate can be increased, better separation in terms of sugars and dye can be achieved.

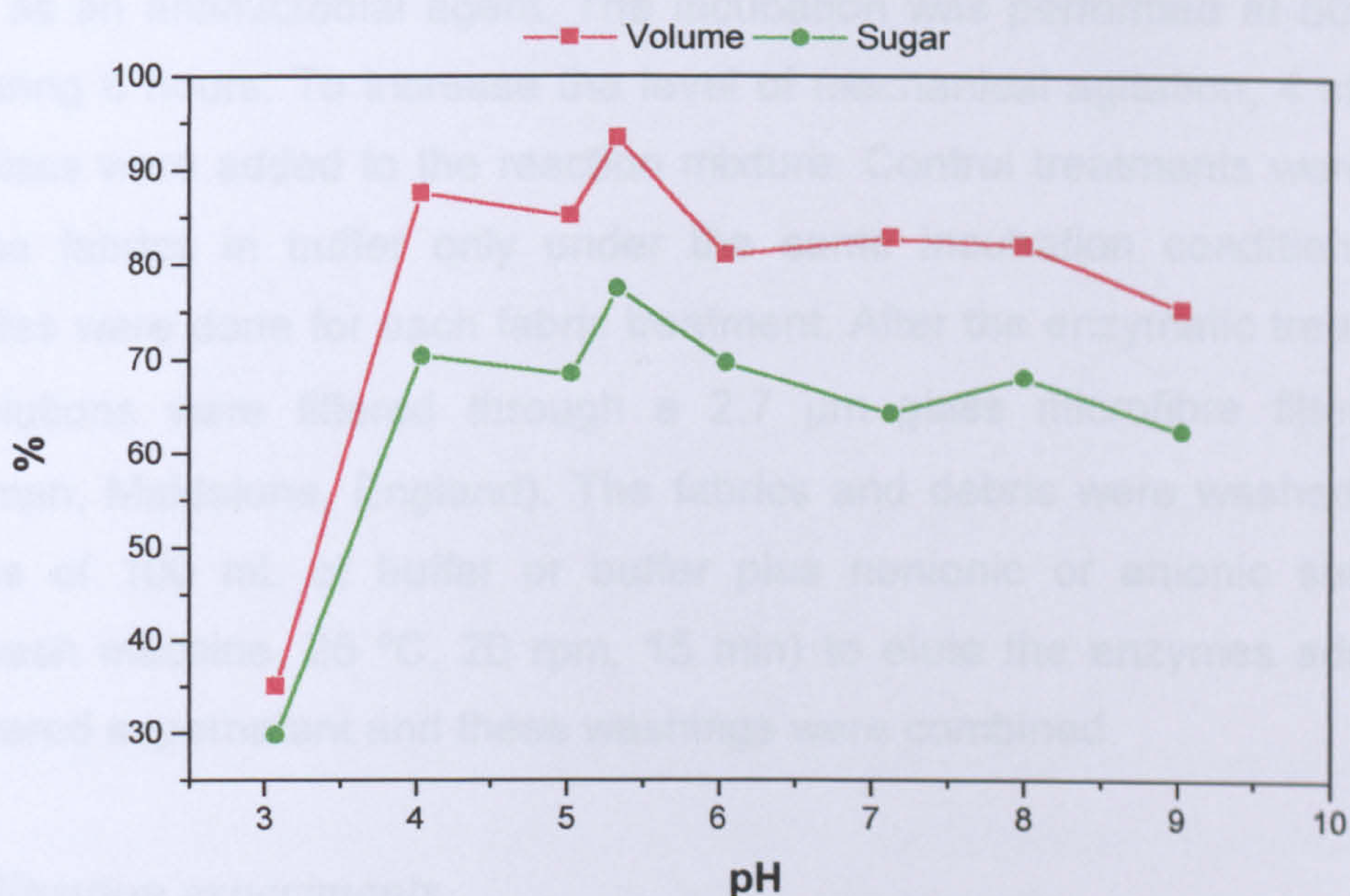


Figure 4.32 Percentages of volume and sugar content of the permeate solution, relative to the initial solution, at different pHs for the hydrolysate of the fabric dyed with indigo.

For indigo-enzyme separation experiments (Figure 4.32), with the exception of pH 3, the ultrafiltration efficiency remained approximately the same throughout the pH range studied. With this dye, better ultrafiltration efficiency (80-95% for permeate volume and 70-80% for sugar remotion) was obtained than with the reactive dye. There was no indigo in the permeate at any pH.

4.5.2 Desorption of cellulases using surfactants and their recovery by ultrafiltration

Enzymatic treatments, desorption of enzymes from treated fabrics and preparation of treatment liquors for ultrafiltration

Fabric samples (10 g) were placed in a pot of a Rotawash machine with 100 mL of the cellulase (TC) solution in acetate buffer (0.1 *M*, pH 5.0). An enzyme load of about 100 mg protein/g of fabric was used with sodium azide (0.02%) being added as an antimicrobial agent. The incubation was performed at 50 °C, 20 rpm during 6 hours. To increase the level of mechanical agitation, 4 stainless steel discs were added to the reaction mixture. Control treatments were made with the fabrics in buffer only under the same incubation conditions. Two replicates were done for each fabric treatment. After the enzymatic treatments, the solutions were filtered through a 2.7 µm glass microfibre filter GF/C (Whatman, Maidstone, England). The fabrics and debris were washed with 5 portions of 100 mL of buffer or buffer plus nonionic or anionic surfactant (Rotawash machine, 25 °C, 20 rpm, 15 min) to elute the enzymes adsorbed. The filtered supernatant and these washings were combined.

Ultrafiltration experiments

The combined solutions (550 mL) obtained from the procedure described above were ultrafiltered using a Prep/Scale™ TFF cartridge (Millipore Corporation, Bedford, MA, USA), containing a 0.09 m^2 polysulphone ultrafiltration membrane with a MWCO of 10 kDa. Before ultrafiltration of the hydrolysate solutions, the ultrafilter was pre-washed with acetate buffer. The ultrafiltration was performed as described in 4.5.1.

The CMC (3.2.11) and FP (3.2.12) activities of the recovered cellulases (retentate) for the three desorbents were determined and expressed in terms of g/L of reducing sugars produced per mg of protein.

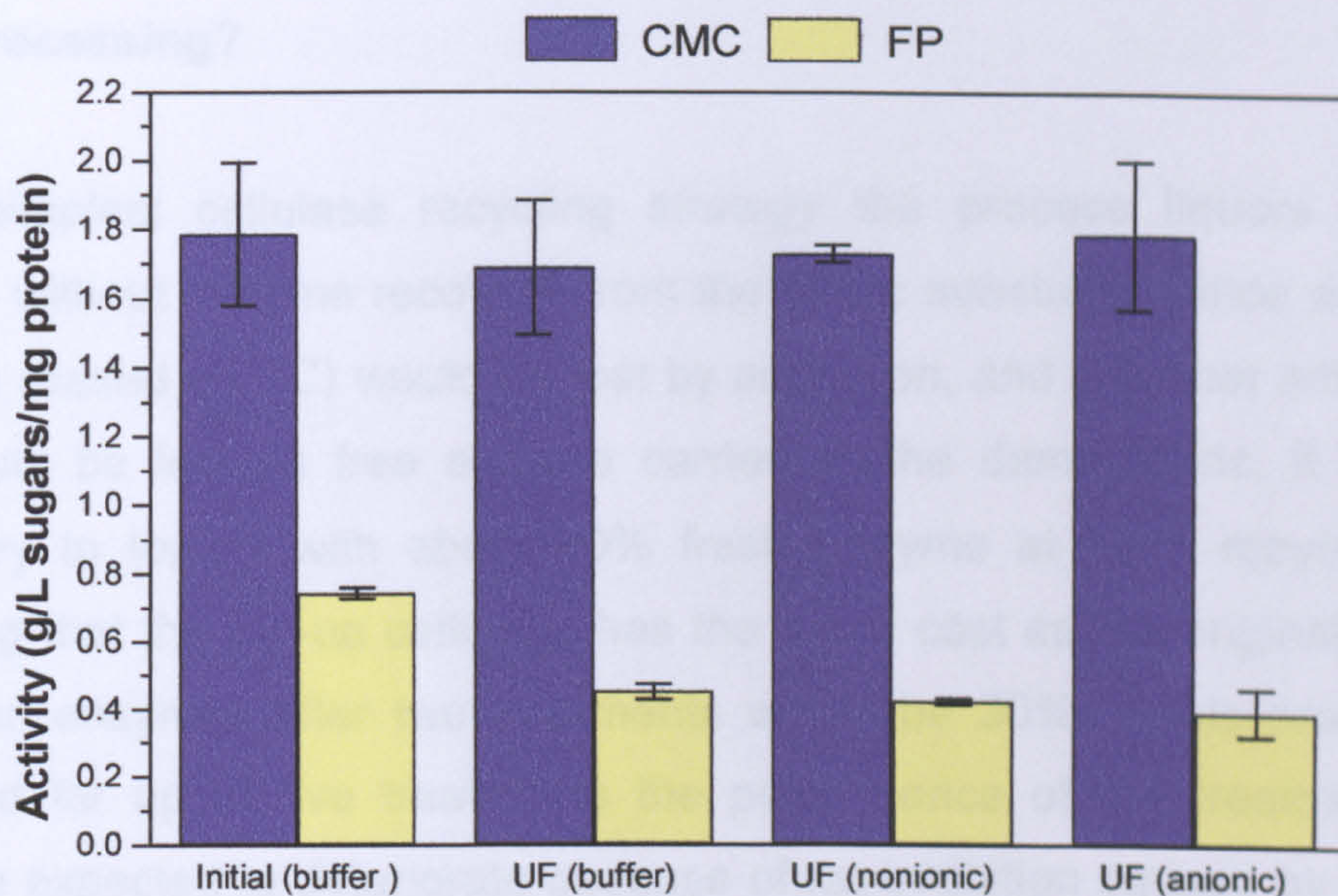


Figure 4.33 Comparison of the CMC and FP activities recovered by ultrafiltration using buffer only and buffer plus nonionic or anionic surfactant (1 g/L) as desorbents. The values in the figure represent the mean of two independent experiments and error bars the standard deviation.

It can be seen from Figure 4.33 that it was possible to recover most of the CMC activity present in the original cellulase sample. The presence of surfactants did not enhance the desorbed activity. The FP activity is, however, partially lost, and this suggests that the CBHs are more sensitive to deactivation. This is probably because CBHII remains irreversibly adsorbed to the cotton substrate. When recycling of a total crude cellulase is carried out, the recycled mixture should therefore be supplemented with a CBHII-rich preparation.
It is surprising that the desorption with anionic surfactant does not cause further deactivation (see 4.4.3 and Figure 4.28) and it appears that ultrafiltration may separate anionic surfactants from cellulase proteins.

4.6 Cost savings or on-cost for recycling cellulases after textile processing?

In the simplest cellulase recycling strategy the process liquors could be recycled without enzyme recovery from the fabric substrate. Since about 35% cellulase (based on TC) would be lost by adorption, and a further amount (say 5%) would be lost as free enzyme carried in the damp fabric, it would be necessary to top-up with about 40% fresh enzyme at each recycling step. Assuming that the top-up cellulase has the same cost as the original, the cost saving on enzymes after two treatments would be 30%. If this process was continued for up to five treatments the performance of the treatment liquor would be expected to deteriorate because of (a) inhibition caused by the build-up in the concentration of end products, and (b) some thermal deactivation effects. The cost saving after five treatments (each with 40% top-up) would be 48%. Probably the extra saving would not be worthwhile in view of expected deterioration in performance, but further work in this area could be considered.

In a more complete cellulase recycling strategy, enzyme recovery from the substrate could be achieved by washing in buffer, but this dilute solution would require concentration by ultrafiltration before further use (4.5.1). Ultrafiltration could also be used to reduce the concentration of end products in recycled supernatant, thereby eliminating inhibition effects. There would, however, still be some enzyme protein losses and slight thermal deactivation at each recycling step.

The capital cost and running costs of an ultrafiltration unit, with adequate capacity for a large-scale textile processing operation (5 L/s throughput) are given in Table 4.13 (information supplied by Mr. Josep Villela of Millipore Ibérica).

Table 4.13 Ultrafiltration costs in thousands of euros (k€).
Millipore Proflux 140 (maximum throughput of 5 L/s).

Cost	k€
Capital cost	130
Membrane cost (p.a.)	22
Running cost* (p.a.)	4
Total annual cost (5 years depreciation)	52
Total annual cost (10 years depreciation)	39

* Includes chemicals for membrane cleaning, energy and labour.

Table 4.14 Ultrafiltration and enzyme costs based on 3% o.w.f. application of an enzyme preparation costing €15.7/kg and 85% protein recovery by UF.

Tonnes of fabric/annum	Enzyme cost (k€/annum)	Ultrafiltration cost (+ 15% enzyme costs) (k€/annum)	
		5 years depreciation	10 years depreciation
50	23.6	55.5	42.5
100	47.1	59.1	46.1
500	235.5	87.3	74.3
1000	471.0	122.7	109.7

These costs are compared in Table 4.14, with those of the estimated enzyme costs for processing various annual tonnages of fabric. The calculations are based on the assumption that 85% of cellulase activity can be recovered from each treatment step (4.5.1). Depending on the depreciation period that applies to the ultrafiltration unit, cost savings become possible when the amount of cellulase-treated fabrics is in the region of 100 to 125 tonnes per annum. Since

there would be spare capacity of the ultrafiltration unit, additional cost savings might be possible from recycling other processing liquors. Further work on pilot scale plant would be necessary to check whether the cost estimates are realistic, but since an annual tonnage of 125 tonnes of cellulase treated fabric would be small for most textile processors, the indications for cost savings are very favourable.

5. SUMMARY AND CONCLUSIONS - SUGGESTIONS FOR FURTHER WORK

5.1 Summary and conclusions

Preliminary recycling experiments (section 4.1) showed that active cellulases remained both in the treatment bath and on the fabric substrate. The adsorption and activity, in the treatment bath, of cellulase enzymes (measured as fabric weight loss) decreased by 80% after 5 recycling steps, although the liberation of soluble reducing sugars was less affected. The decrease in adsorption and activity could have been due to end-product inhibition, thermal and or mechanical deactivation or to the loss of some components of the cellulase complex, possibly by preferential or irreversible adsorption to the cotton substrates.

The end-product inhibition studies (section 4.2) revealed that cellobiose is a stronger inhibitor than glucose, but its effects on the activity (weight loss) were not enough to account for the losses observed in the preliminary recycling experiments. There was a 25% loss of activity in the presence of 5 g/L of cellobiose which is still greater than the possible cellobiose concentration after five fabric treatments.

Measurement of the kinetics of the hydrolysis of cellobiose by β-glucosidase present in the TC preparation of *T. reesei*, indicated that this enzyme is not sufficiently active to hydrolyse all of the cellobiose produced during the consecutive treatments. There was therefore a build-up of cellobiose and glucose concentrations in the recycled treatment liquors. This problem could, however, be overcome by reducing their concentration using ultrafiltration.

Measurement of thermal and mechanical deactivation of cellulases (section 4.3) showed that cellulases are denatured by temperatures above 60 °C and that

EGs are more thermostable than CBHs. The agitation levels used (typical of textile processing) did not cause significant cellulase deactivation effects. Incubating cellulase (TC) solutions at 50 °C for 5 h (equivalent to 5 treatment cycles) reduced their activity by 20%.

The studies of adsorption of individual cellulase components by FPLC (section 4.4.1) showed that there seems to be some depletion of CBHII in the supernatant after treatments on cotton fabrics. This may indicate that CBHII is irreversibly adsorbed to cotton. EGII also showed a similar behaviour but this could only be detected in the EG-rich preparation. These results suggest that there is a need to supplement the recycled liquor with selected pure cellulase components in order to maintain a constant composition and thereby to deliver, reproducibly, the desired effects to treated fabrics. Alternatively cellulases could also be recovered from treated fabrics by washing with buffer and concentrating the solution by ultrafiltration. This gave an enzyme protein recovery (from supernatant plus fabric) of about 85% (4.5.1).

It can be concluded that cellulase activity losses result from end-product inhibition, thermal deactivation and preferential adsorption of CBHII. The loss of CBHII from the total cellulase complex appears to be the most serious of these effects with regard to maintaining reproducible fabric treatments.

A study of the adsorption/desorption behaviour of purified EGV from *H. insolens* and CenA from *C. fimi* with and without their CBDs showed that the EGV adsorption (with or without its CBD) was fully reversible and that its activity on cotton cellulose is not much affected by the presence of its CBD. CenA adsorption was irreversible although its reversibility (especially at higher agitation rates) improved on removal of its CBD. This did not reduce its hydrolytic activity towards cotton.
Other authors have reported that CBHI (ex *T. reesei*) adsorption is reversible (Linder and Teeri, 1996) but that its CBD is necessary for its activity on crystalline cellulose (Kotiranta *et al.*, 1999). Kotiranta *et al.* (1999) have also

shown that the presence or absence of the CBD of EGII (from *T. reesei*) does not much affect its hydrolytic activity on insoluble substrates. These findings suggest that it may be possible to tailor effective cellulase compositions, for cotton finishing, which contain only cellulase components that are adsorbed reversibly. Such preparations would be ideal for recycling since their composition would remain constant through repeated treatments.

Although ultrafiltration was used successfully to reduce the concentration of sugars in treatment liquors, the presence of dyes, especially vat dyes (indigo) in these liquors proved to be a serious problem for recycling because ultrafiltration could not remove dye adsorbed on the cellulase protein. Unfortunately this means that recycling of cellulases in enzymatic stonewashing (the most important cellulase-finishing process) may not be possible, unless an alternative approach to the backstaining problem can be found. It may be that cellulase compositions containing only reversibly adsorbed components would also be beneficial in this context.

Cost estimates suggested that when more than 100 to 125 tonnes per annum of cellulosic fabric are being treated with cellulases (a very small tonnage for most textile processors) the savings made by recycling enzymes would exceed the capital and running costs of the ultrafiltration unit required for their recovery and concentration.

5.2 Suggestions for further work

- Thermal deactivation of TC (*T. reesei*) after 5 h at 50 °C accounted for about 20% activity loss. It is suggested that this could be reduced by the addition of protein stabilizers at each recycling step. Commercial preparations are known to contain stabilisers to prolong shelf life but the

role of such stabilisers during processing has not been reported in the literature.

- It has been suggested that CBHII depletion accounts for much of the activity loss on recycling TC from *T. reesei*. Unequivocal evidence for this is required before attempting to top-up the CBHII content of recycled liquors. It is therefore proposed, that in further work, depletion of CBHII should be confirmed by FPLC analysis of treatment liquors after several recycling steps. Additionally the composition of cellulase desorbed from the cotton substrates should also be analysed by FPLC to confirm or deny that the strongly adsorbed fraction is rich in CBHII.

- If the depletion of CBHII is confirmed, further experiments should be carried out in which the depleted treatment liquor is topped up with CBHII and its activity on cotton fabric is compared with that of the original TC preparation.

- To avoid the problem of TC deactivation through irreversible adsorption of specific components, it is suggested that cellulase compositions (to achieve specific fabric finishing effects) can be designed to contain only components that are adsorbed reversibly. Some effects are already achieved with cellulases containing only EGs and these should first be tested in recycling experiments. Further work in this area might involve determination of the adsorption/desorption and activities of purified major components (CBHI, CBHII, EGI, EGII) with and without their CBDs.

- The problem of backstaining with recycled treatment liquors containing indigo (or other vat dyes) might also be alleviated by using only reversibly adsorbed cellulases. The activities of cellulase/indigo complexes need to be confirmed.

- Alternative approaches to ultrafiltration for recovering cellulases from treatment liquors might prove to be more cost effective. Methods already described in patents (Fujishima *et al.* 1988; Woodward and Ridge, 1989) should be tested in the context of cotton processing.

- Cost estimates suggested that recycling treatment liquors could lead to savings of 30-48% of the enzyme costs but further work is needed to confirm that this is achieved without unacceptable deterioration in performance.

- When cellulases are recovered from treated fabrics by washing with buffer followed by concentration using ultrafiltration, cost savings are estimated to be possible when the amount of cellulase-treated fabric in in the region of 100 to 125 tonnes per annum. Further work on pilot scale plant (including pilot scale ultrafiltration) is needed to confirm that these cost estimates are realistic.

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APPENDICES

APPENDIX A: Raw data

Table I Cellulase (TC from *T. reesei*) activity (in terms of fabric weight loss) towards cotton fabrics in the presence of increasing concentrations of glucose and cellobiose. Values in brackets represent the standard deviation of two independent experiments.

Sugar	Concentration (g/L)	Weight loss (%)
Glucose	0	2.86 (0.07)
	5	2.66 (0.06)
	10	2.55 (0.05)
	20	2.72 (0.17)
	30	2.23 (0.05)
Cellobiose	0	2.86 (0.07)
	5	2.12 (0.01)
	10	1.77 (0.03)
	20	1.59 (0.00)
	30	1.55 (0.03)

Table II Effect of pre-incubation temperature and time on cellulase (TC from *T. reesei*) activity measured as soluble reducing sugars liberated during hydrolysis of cotton fabrics. Values in brackets represent the standard deviation of two independent experiments.

Temperature (°C)	Time (h)	[Reducing sugars] (g/L)
-	0	857.6 (61.8)
37	1	1016.7 (64.3)
	2	946.9 (29.3)
	3	984.3 (84.4)
	4	932.9 (15.86)
	5	863.2 (213.7)
50	1	871.6 (127.7)
	2	742.0 (88.0)
	3	751.9 (17.2)
	4	791.9 (31.8)
	5	681.0 (132.0)
60	1	296.4 (55.9)
	2	170.9 (17.2)
	3	74.3 (7.8)
	4	44.1 (29.9)
	5	85.3 (16.1)
70	1	43.7 (6.8)
	2	27.9 (32.4)
	3	26.6 (30.6)
	4	57.03 (80.7)
	5	58.6 (24.6)
80	1	28.3 (7.1)
	2	24.3 (28.5)
	3	0.0 (0.0)
	4	26.6 (37.6)
	5	14.3 (20.3)

Table III Effect of pre-incubation temperature and time on cellulase (TC from *T. reesei*) adsorption, measured as protein adsorbed on cotton fabrics. Values in brackets represent the standard deviation of two independent experiments.

Temperature (°C)	Time (h)	mg protein/g fabric
-	0	1.97 (0.23)
37	1	2.91 (0.13)
	2	2.88 (0.11)
	3	2.68 (0.02)
	4	2.76 (0.08)
	5	2.96 (0.06)
50	1	2.23 (0.01)
	2	2.54 (0.03)
	3	2.40 (0.28)
	4	2.27 (0.13)
	5	2.63 (0.22)
60	1	2.33 (0.21)
	2	2.26 (0.16)
	3	2.51 (0.26)
	4	3.07 (0.07)
	5	2.67 (0.20)
70	1	3.18 (0.08)
	2	3.56 (0.03)
	3	3.67 (0.42)
	4	3.08 (0.23)
	5	3.31 (0.26)
80	1	2.98 (0.05)
	2	3.21 (0.08)
	3	3.19 (0.05)
	4	3.39 (0.69)
	5	3.55 (0.71)

Table IV CMC and FP activities (measured as liberation of soluble reducing sugars) by cellulase preparations from *T. reesei* after thermal and mechanical treatment. Values in brackets represent the standard deviation of two independent experiments.

Cellulase preparation	Treatment	[Reducing sugars] (g/L)	
		CMC	FP
TC	1	0.956 (0.021), 0.808	0.701 (0.06), 1.256
	2	0.931 (0.073), 0.796	0.649 (0.015), 1.004
	3	0.972 (0.030) 0.818	0.693 (0.000), 1.198
	4	0.863 (0.013), 0.746	0.636 (0.050), 1.162
	5	0.984 (0.007), 0.820	0.678 (0.009), 1.278
	6	0.885 (0.022), 0.816 (0.023)	0.625 (0.036), 1.171 (0.028)
	7	0.905 (0.021), 0.771	0.600 (0.001), 0.796
EG-rich	1	1.332 (0.027), 0.868*, 0.712**	0.883 (0.016), 0.646*, 0.453**
	2	1.330 (0.016)	0.880 (0.014)
	3	0.967* (0.035), 0.933**	0.597* (0.024), 0.669**
	4	1.424 (0.027)	0.802 (0.082)
	5	1.316 (0.022)	0.872 (0.020)
	6	0.994* (0.013), 0.911** (0.001)	0.610* (0.011), 0736** (0.066)
	7	0.933* (0.040), 0.849*	0.531* (0.008), 0.616**
CBH-rich	1	0.700 (0.029), 0.658* (0.011)	0.545 (0.031), 0.447* (0.025), 0.900**
	2	0.707 (0.032)	0.526 (0.032)
	3	0.605* (0.042), 0.511**	0.437* (0.024), 0.867**
	4	0.716 (0.040)	0.581 (0.058)
	5	0.704 (0.015)	0.483 (0.069)
	6	0.573* (0.013), 0.534** (0.014)	0.410* (0.011), 0.849** (0.010)
	7	0.581* (0.095), 0.450**	0.326* (0.000), 0.422**

Table V Total, insoluble and soluble fabric weight loss produced by purified endoglucanases (EGV from *H. insolens* and CenA from *C. fimi*) and their isolated cores using high level of agitation (Rotawash machine, 50 °C, 5 discs, 40 rpm, 1 h) and low level of agitation (shaker bath, 50 °C, 125 rpm, 24 h) on the enzymatic treatments of cotton fabrics (1.5 mg protein/g fabric). Values in brackets represent the standard deviation of two independent experiments.

Treatment	Enzyme	Total weight loss (%)	Insoluble weight loss (%)	Soluble weight loss (%)*
Rotawash	EGV	3.9 (0.2)	1.7 (0.2)	2.2
	EGV core	2.4 (0.1)	1.3 (0.1)	1.9
	CenA	1.0 (0.1)	0.7 (0.1)	0.3
	CenA core	1.1 (0.1)	0.6 (0.0)	0.5
Shaker	EGV	0.2 (0.1)	0.0 (0.0)	0.2
	EGV core	0.1 (0.0)	0.0 (0.0)	0.1
	CenA	0.0 (0.0)	0.0 (0.0)	0.0
	CenA core	0.0 (0.0)	0.0 (0.0)	0.0

*Soluble weight loss was obtained by difference.

Table VI Reducing sugars produced during the enzymatic hydrolysis (Rotawash machine, 50 °C, 20 rpm, 3.6 mg protein/g fabric, 1 h) of cotton fabrics in presence of buffer only and buffer plus nonionic or anionic surfactant (1 g/L). Values in brackets represent the standard deviation of two independent experiments.

Cellulase system	Enzymatic hydrolysis	[Reducing sugars] (g/L)
TC from *T. reesei*	Buffer only	1.409 (0.027)
	Buffer plus nonionic surfactant	1.516 (0.031)
	Buffer plus anionic surfactant	0.976 (0.004)
TC from *H. insolens*	Buffer only	0.220 (0.002)
	Buffer plus nonionic surfactant	0.220 (0.025)
	Buffer plus anionic surfactant	0.046 (0.006)

Table VII Reducing sugars produced during the enzymatic hydrolysis (Rotawash machine, 50 °C, 20 rpm, 1 h) of cotton fabrics using "fresh" cellulases and cellulases desorbed from treated fabrics with buffer only and buffer plus nonionic or anionic surfactant (1 g/L). Values in brackets represent the standard deviation of two independent experiments.

Enzymatic hydrolysis	[Reducing sugars] (g/L)
"Fresh" cellulases (buffer only)	1.299 (0.015)
Cellulases desorbed with buffer	0.412 (0.009)
Cellulases desorbed with nonionic surfactant in buffer	0.438 (0.028)
Cellulases desorbed with anionic surfactant in buffer	0.229 (0.009)

APPENDIX B: Chromatograms

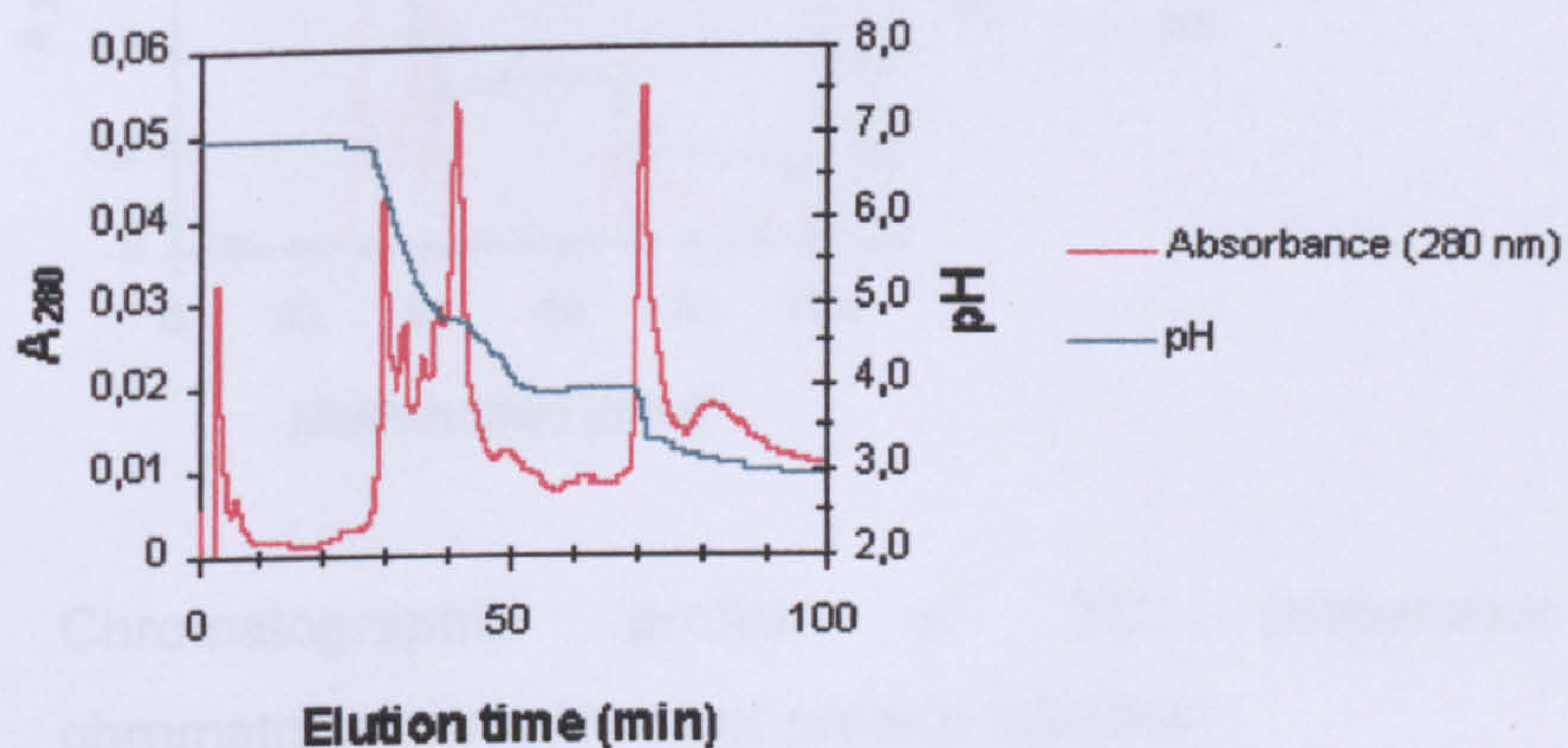

Figure I Chromatographic profile of TC preparation using chromatofocusing (0.1 mg of protein injected).

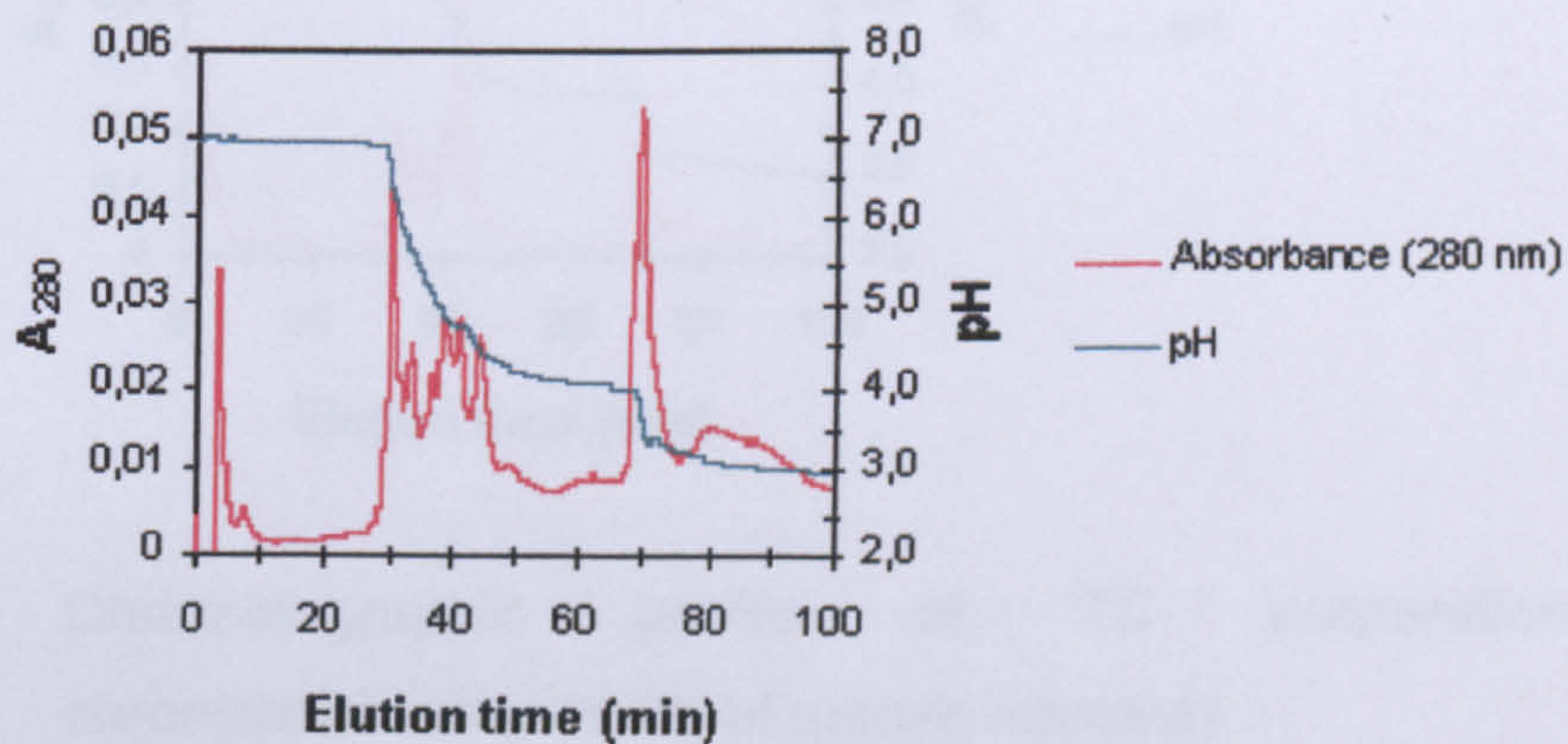

Figure II Chromatographic profile of TC preparation using chromatofocusing (0.1 mg of protein injected).

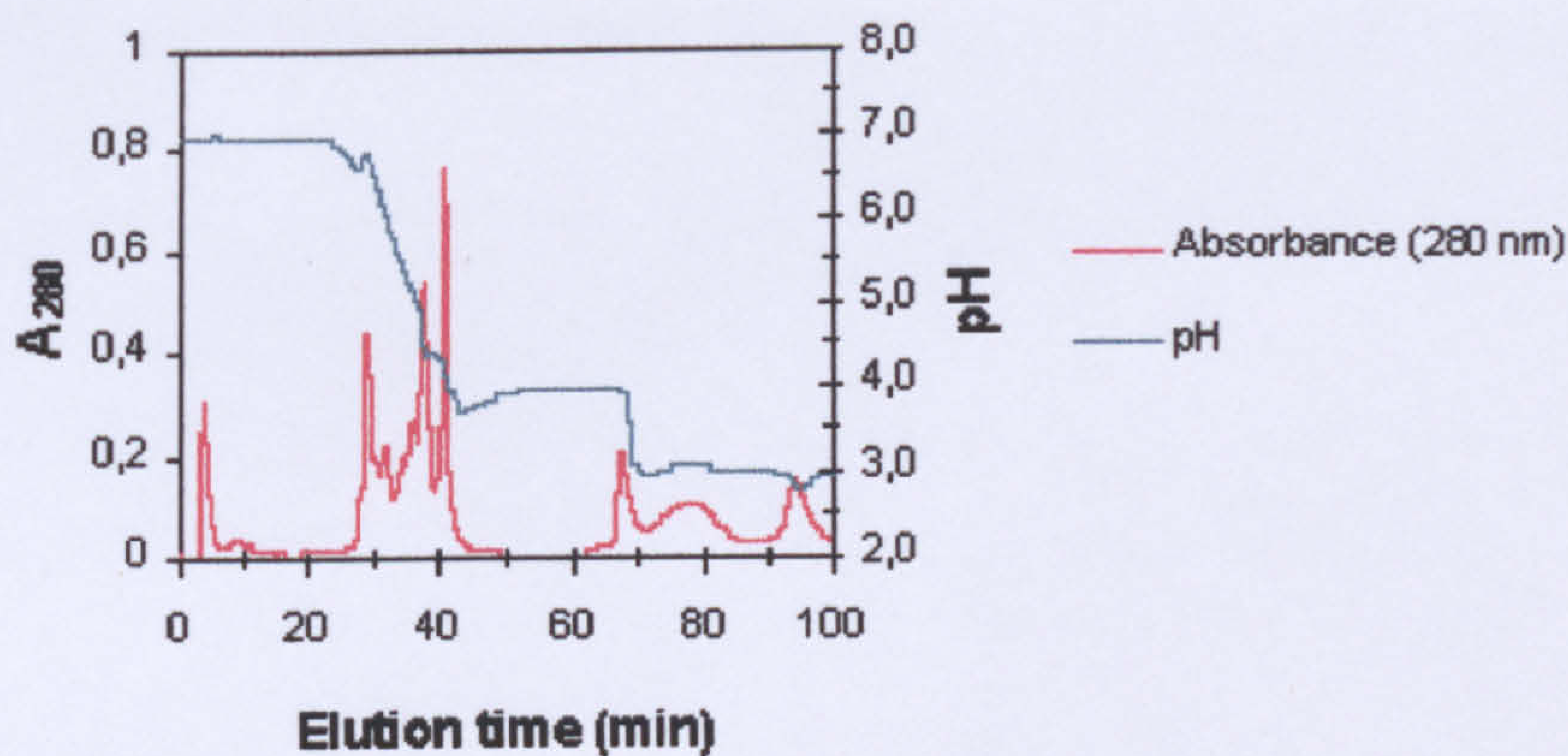

Figure III Chromatographic profile of TC preparation using chromatofocusing (1 mg of protein injected).

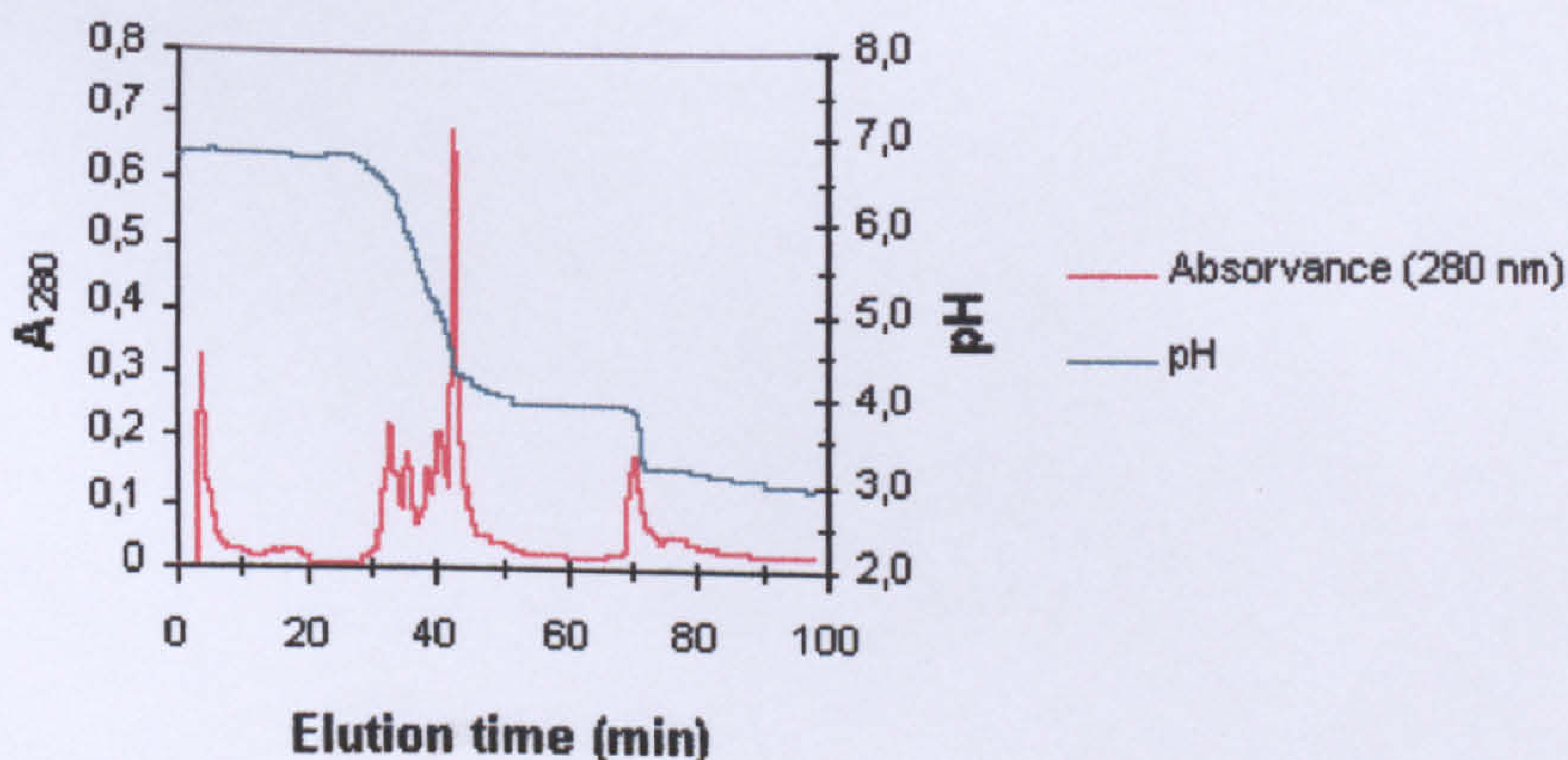

Figure IV Chromatographic profile of TC preparation using chromatofocusing (1 mg of protein injected).

The chromatograms obtained separately prepared samples of TC were reproducible with respect to the elution times and pH ranges of the main cellulase components but the peak areas varied considerably from sample to sample. It was assumed that this would not be a problem when comparing samples from the same solution before and after adsorption on cotton fabrics.