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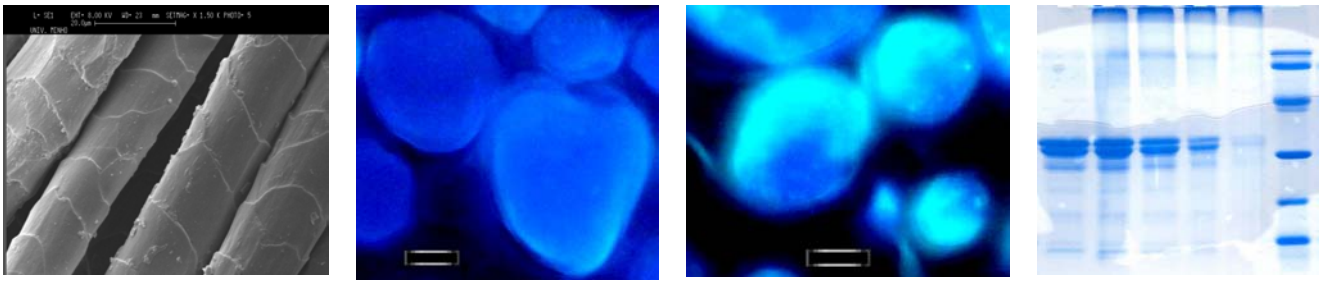

Enzymatic Treatment of Wool with Modified Proteases

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Guimarães, 2005

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Enzymatic Treatment of Wool with Modified Proteases

Dissertation presented for PhD degree in Textile Engineering

at University of Minho

Dissertação apresentada para obtenção do grau de Doutor em Engenharia

Têxtil pela Universidade do Minho

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The research described in this thesis was performed at the Departamento Têxtil da Universidade do Minho, Campus de Azurém and was financially supported by *Fundação para a Ciência e Tecnologia* by means of a PhD grant (PRAXIS XXI/BD/1292/2000) and by EU funding for *PROTEX project* G1RD-CT-2002-00695.

À minha familia,

pela beleza da Vida,

da Partilha e do

Amor!

ACKNOWLEDGEMENTS

I am greatly indebted to many people whose support, encouragement and friendship made this thesis possible.

First and foremost I would like to express my sincere gratitude to Dr. Artur Cavaco-Paulo, my supervisor, for all his patience, support and guidance throughout the years, and for the opportunity to attend several scientific meetings and conferences.

I would like to express my sincere thanks to my former and present colleagues at the *Laboratório de Investigação em Acabamentos*, namely Helena, Cesar, Tzanko, Filipa, Zille, O´Neill, Patrícia, Florentina, Carla, Teresa, Carlos, Andreia, Rita, Su, Ana Felix and our "Erasmus" students. Thank you for creating a nice working atmosphere in the lab, for all the company and friendship, and for all the fun! Special thanks to Florentina, Filipa and Ana for their help and suggestions with this thesis.

I am grateful to all my friends, especially Luis, Cristininha and Isabel, for believing and always being there through these years!

I would like to thank the *Fundação para a Ciência e Tecnologia* for their financial support.

I do not have enough words to express my gratitude to my parents, brothers, sisters and lovely nephews and nieces for their love and support in this endeavour.

And finally, I want to thank my husband, Paulo, and my little daughter, Joana, for showing me "the meaning of life". Thank you Paulo for your understanding and for all the constant support and encouragement, for your deep love, confidence and serenity making me believe in this day. I also want to thank you for being a superb father, assisting and caring for Joana when I had to be away and finally, for your Canadian side, in helping with the English language. To my little baby, thank you for your pure smile, tender hugs, strong character and for showing me the beauty of life…and…the eternity of night.

April 2005

ABSTRACT

The tendency of wool to felt and shrink is mainly due to its scaly structure. The chlorine-Hercosett is the most widespread process used to modify the scales of wool fibres with the purpose of providing resistance to felting and shrinkage. There have been many attempts to replace this chlorine process by an environmental friendly enzymatic process that would similarly degrade the scales. However, although proteases are large molecules, their attack is not only limited to the scales; they penetrate inside the fibre causing unacceptable weight and strength loss. It is believed that if the proteases are chemically modified in order to increase their molecular weight, then they will act just on the surface of the fibres, thus providing wool with anti-shrinking behaviour, which is the main idea of this research.

In this work the screening of the attack of the proteolytic enzymes inside the fibre was made by means of several techniques. Among them, special attention was paid to the study of the protein adsorption inside the wool fibres. It was demonstrated that the penetration of protein (measured as the maximum adsorption capacity in g protein/g wool) was higher when the wool was previously subjected to a surfactant washing and bleaching. Furthermore, it was observed that the diffusion of the proteases into wool was dependent on their size. The free enzyme penetrated into wool fibre cortex while the modified enzyme, with a bigger size, was retained at the surface, in the cuticle layer. It was also confirmed that the diffusion of proteases was facilitated by the hydrolytic action. Scanning electron microphotographs were also used to observe the intensity of the proteolytic attack.

Some techniques of increasing the proteases molecular weight were attempted, namely the covalent crosslinking method using the bifunctional reagent glutaraldehyde. It was observed that the low amount of free lysine residues available in the protease for crosslinking was affecting the process. A more successful technique was attained by covalently coupling the enzyme to a soluble-insoluble polymer of high molecular weight. An enzyme conjugated to such a carrier may be used as a catalyst in its soluble form and then be recovered via the insoluble state. Moreover, this system overcomes the problem of the non accessibility of the enzyme to the macromolecular substrate, wool, whilst in the soluble state. When comparing to the native enzyme, the immobilized form presented a lower specific activity towards high molecular weight substrates but a higher thermal stability at all temperatures tested. It also exhibited a good storage stability and reusability, which makes this enzyme conjugate quite interesting from an industrial point of view. Wool fabrics were treated with the immobilized serine protease using harsh conditions and subjected subsequently to several machine washings, after which they presented a significant lower weight loss than wool treated with the native enzyme, in the same conditions. Using a moderate enzymatic treatment, a reduction to about half of the initial area shrinkage was attained, both for free and immobilized enzymes. However, the immobilized Esperase presented 92% of the original tensile strength resistance while native Esperase kept only 75% of its initial resistance.

The coupling of the protease to the polymer, Eudragit S-100, was optimized by using experimental design techniques. This optimization strategy allowed for an enzyme conjugate wherein the enzyme was covalently crosslinked to the polymer, with high activity yield and high operational stability at 60ºC.

All these results prove that modified proteases attained by this immobilization method, using a soluble-insoluble polymer of high molecular weight, can be a promising alternative for wool bio-finishing processes at an industrial level, since it is an effective way of removing wool scales and can be an environmental friendly option to the conventional chlorine treatments. This process needs to be further characterized for its complete understanding and optimization.

RESUMO

A tendência da lã para feltrar e encolher é devida, principalmente, à sua estrutura em escamas. O tratamento anti-feltragem normalmente utilizado para modificar as escamas das fibras de lã utiliza cloro, pelo que, várias tentativas têm sido levadas a cabo para substituir este processo por um processo enzimático amigo do ambiente. Estes processos recorrem à utilização de proteases que, apesar do seu tamanho, atacam não só a cutícula mas também penetram rapidamente dentro da fibra, provocando perdas de peso e resistência nas fibras, inaceitáveis do ponto de vista comercial. Assim sendo, este trabalho teve como principal objectivo o desenvolvimento de técnicas que permitissem modificar as proteases, aumentando o seu peso molecular e restringindo, deste modo, o seu ataque à superfície da fibra, removendo apenas a cutícula.

Várias técnicas foram utilizadas para a monitorização do ataque das enzimas proteolíticas ao interior da fibra, como por exemplo a adsorção de proteína nas fibras. Verificou-se que a penetração de proteína (medida como a máxima capacidade de adsorção em g proteína/g lã) foi superior quando a lã foi sujeita a um pré-tratamento de lavagem alcalina com surfactante e posterior branqueamento. Foi ainda constatado que a difusão das proteases na lã depende do seu tamanho. A enzima livre penetrou no córtex da fibra de lã enquanto que a enzima modificada, de maior tamanho, ficou retida à superfície, na cutícula. Foi também confirmado que a difusão das proteases no interior da fibra foi facilitada pela sua acção hidrolítica. A técnica de microscopia electrónica foi também utilizada para observar a intensidade do ataque proteolítico.

Para aumentar o peso molecular das proteases foram tentadas algumas estratégias, entre as quais o método de ligação covalente ao glutaraldeído, um reagente bifuncional. Demonstrou-se que o baixo teor em resíduos lisina da protease, disponíveis para ligação, afecta o processo. Uma técnica mais eficaz foi a ligação covalente da enzima a um polímero solúvel-insolúvel de elevado peso molecular. De facto, as enzimas ligadas a este tipo de suportes podem ser utilizadas como catalisadores na sua forma solúvel sendo posteriormente recuperadas no seu estado insolúvel. Para além disso, este tipo de imobilização contorna os problemas da não acessibilidade da enzima ao seu substrato macromolecular, a lã. A enzima imobilizada, quando comparada com a enzima nativa, apresentou uma actividade específica menor para substratos de elevado peso molecular, mas uma estabilidade térmica superior, a todas as temperaturas testadas. A protease imobilizada no polímero entérico exibiu ainda uma boa estabilidade de armazenamento e boa reutilização, o que torna este conjugado enzimático muito interessante do ponto de vista industrial. Foram tratados tecidos de pura lã em condições severas com a protease imobilizada, que foram depois sujeitos a várias lavagens domésticas à máquina. Os tecidos tratados com a enzima modificada apresentaram uma menor perda de peso do que a lã tratada com a enzima nativa, nas mesmas condições. Usando um tratamento enzimático moderado, verificou-se uma redução para cerca de metade no encolhimento da lã, para ambas as enzimas. No entanto, a Esperase imobilizada conservou cerca de 92% da sua resistência à tracção enquanto a Esperase nativa apresentou apenas 75% da sua resistência original.

O processo de acoplamento da protease ao polímero, Eudragit S-100, foi optimizado usando técnicas de desenho experimental. Esta estratégia de optimização permitiu obter um conjugado no qual a enzima se encontra covalentemente ligada ao polímero, com elevado rendimento em actividade e uma alta estabilidade operacional a 60ºC.

Os resultados obtidos mostram que as proteases modificadas pelo método de imobilização descrito neste trabalho, (usando um polímero solúvel-insolúvel de elevada massa molecular), podem ser uma alternativa promissora para os processos de bio-acabamento da lã, uma vez que, constituem um modo eficaz de remover as escamas da lã, podendo ser uma opção ambientalmente aceite para substituir os tratamentos convencionais com cloro. Este processo necessita, no entanto, de ser melhor caracterizado para a sua completa compreensão e optimização.

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CHAPTER 1

GENERAL INTRODUCTION

"The most beautiful thing we can experience is the mysterious.
It is the source of all true art and all science. He to whom this emotion is a
stranger, who can no longer pause to wonder and stand rapt in awe,
is as good as dead: his eyes are closed."

Albert Einstein (1879-1955)
German physicist

1. GENERAL INTRODUCTION

The utilization of enzymes in the textile industry has been known and applied commercially for many years, principally in cellulosic fibres, like the use of amylases for desizing of cotton and cellulases for indigo abrasion on denim (Cavaco-Paulo and Almeida 1994; Cavaco-Paulo *et al*. 1996; Ericksson and Cavaco-Paulo 1998). However, for protein fibres, there are other possibilities for enzyme application, including the use of proteases for wool and silk processing (Nolte *et al*. 1996; Heine and Höcker 1995; Riva *et al*. 1999) and for the surface modification of cashmere fibres (Hughes *et al*. 2001), for instance.

Wool is a complex natural fibre composed mainly of proteins (97%) and lipids (1%), consisting of two major morphological parts: the cuticle and the cortex. The former is composed of overlapping cells (scales) that surround the latter. This scaly structure of wool is responsible, to a great extent, for the tendency of wool to felt and shrink (Heine and Höcker 1995; Feughelman 1997). Chlorination is a commonly used process to modify the scales of wool fibres with the purpose of providing resistance to felting and shrinkage. There have been many attempts to replace this chlorine process by an environmental friendly enzymatic process that would similarly degrade the scales (Nolte *et al*. 1996; Heine and Höcker 1995; Silva and Cavaco-Paulo 2003; Cortez *et al*. 2004). However, despite proteases are large molecules, their attack is not only limited to the scales, causing unacceptable weight and strength loss to the fibres.

It is believed that if the proteases are chemically modified in order to increase their molecular weight, their attack would be restricted only to the surface

of the fibres, thus removing the cuticle, which is the main purpose of this doctoral thesis.

In agreement with the aims defined for the present thesis, in the general introduction corresponding to chapter 1, a brief bibliographic revision is made concerning the most relevant topics related to the use of proteases for wool bio-finishing. The second part contains the major results attained in the scope of this thesis. The third part presents a general discussion, the major conclusions and gives some perspectives for continuing the work in this research field.

Thus, the organization of this thesis comprehends 3 major parts:

- 1st part: Theoretical considerations about enzymatic wool treatments and aims of the work – Chapter 1
- 2nd part: Major results, in the scope of this thesis. This section contains the experimental procedures, as well as the attained results and respective discussions – Chapters 2, 3, 4, and 5.
- 3rd part: General discussion, conclusions and future perspectives – Chapters 6 and 7.

1.1 PROPERTIES AND STRUCTURE OF WOOL FIBRE

The world's animal population has many species with bodies covered by hair, fur or wool. Mankind has the body least equipped to live in the world's varied climatic conditions, but man has used his intellect and enterprise to compensate for this. Primitive man protected his body with animal skins, and he learned to appreciate the merits of alternative furs according to fibre length, fibre fineness (or fibre diameter) and fibre density of the pelt. Innovative man eventually looked beyond furs to other fibrous materials, and he began to prepare them and make garments to suit his needs. In the world's hot and temperate zones, the fibres used were usually cellulosic or vegetable-based: cotton, linen, jute, ramie and hessian. These fibres are all vegetable in origin and have a common chemistry. Cellulosic fibres grow readily in hot climates, and the resultant garments were designed appropriately for wear in hot weather. In colder regions, nomadic tribesmen combed their animal flocks as they moulted each spring, and spent the long winter nights spinning and weaving the soft, woolly fibres into garments which would keep them warm and dry throughout the cold winter season ahead.

In the late 18th Century, the Industrial Revolution began a movement which took the textile industry from the home into the factory. Machines were invented to carry out processes which for countless generations had been carried out by hand. The machines and factories developed an insatiable demand for fibres, and an international trade in textile fabrics began to develop. All textile fabrics manufactured prior to 1884 were made of the natural fibres: wool, silk, cotton and linen. The most recent evolution in the textile industry has been the introduction of man-made fibres (Araújo and Melo e Castro 1984). Since the invention of synthetic fibres

many fabrics have been manufactured entirely of these fibres or mixed with natural ones. The first group of man-made fibres which are still widely in use were "regenerated" from naturally occurring products. For example, cellulose from trees is regenerated into fibrous cellulose to produce viscose rayon. The next evolutionary step was to completely synthesize the fibrous material. The oil industry yielded the base products for the synthesis of nylons, acrylic and polyester fibres. These are termed "man-made synthetic fibres".

Thus, the contemporary consumer is able to choose from a wide array of fibres, which may be classified as follows (Ferreira Neves 1982; EPA 1996):

- **Cellulosic fibres** - These are natural fibres of vegetable origin, like cotton, linen, jute, ramie, hessian and sisal.
- **Protein fibres** - These are natural fibres of animal origin, like wool (Sheep), alpaca (Alpaca), mohair (Goats), cashmere (Goats), angora (Rabbit), camel (Camel), vicuna (Vicuna) and silk (Silkworm).
- **Man-made fibres** - These are fibres produced by man. For many years textile technologists endeavoured to produce fibres with similar characteristics to natural ones. Now they are attempting not to imitate natural fibres, but to create fibres with characteristics which are distinct from these. Synthetic fibres now are playing a major role in the textile industry, due partly to the great variety of moderately priced fabrics which can be made from them.

Table 1.1 – Some examples of the man-made fibres (based on Ferreira Neves 1982)

Regenerated cellulose fibres	*Synthetic fibres*
• Cellulose Acetate • Cellulose Triacetate • Viscose Rayon • Polynosic	• Polyamide • Polyacrylic • Polyester • Polyethylene • Polypropylene • Polyvinylchloride

1.1.1 THE NATURAL PROPERTIES OF WOOL

Wool, one of the oldest textile fibres known, has survived the test of time because of its unique natural properties (Table 1.2). The basic characteristics that wool possessed in the Stone Age are still the fundamental qualities that make wool unique in this century. Today there are many other textile fibres, but science as yet to produce another fibre containing all the natural properties of wool (Fact Sheet 2005; FAO 1995).

Hence, wool is a remarkable renewable resource with exceptional properties - cool in summer, warm in winter and in a variety of weights suitable for both apparel and interior fibre applications. Wool has excellent flame-resistant properties. This factor is of importance in industrial safety garments and in institutions. Legislation on children's night clothes has been a reminder of its value in domestic clothing also. The natural flame resistance of wool is inherent - it will not wash out or decrease in effectiveness with age. Consumers' perception of the benefits of using natural products has stimulated interest in industrial uses of wool (Glaser 1996). However, the laundry and durability performance of wool is inferior to

synthetics; a factor which is a key selling point for synthetic fibre manufacturers and the cause of much research and development work in the wool industry.

Table 1.2 – Brief description of wool's natural properties

• Insulation	Because it absorbs moisture vapour, wool clothing provides superior comfort in both hot and cold weather.
• Health	Because wool has the ability to insulate against heat and cold, it protects against sudden changes of temperature, and it lets your body breathe.
• Water Repellence	While wool can absorb moisture, it repels liquids.
• Fire Resistance	Wool is naturally safe. It does not have to be specially treated to become non-flammable. Wool does not melt when burned, and so will not stick to the skin and cause serious burns.
• Resilience	Wool's natural elasticity, greater than that of any other fibre, makes it comfortable to wear because it fits the shape of the body. Wool can be twisted, turned and stretched, and yet it returns to its natural shape.
• Versatility	Wool fabric, knitwear and carpets are made from a wide range of wool types varying from extra fine for suits and knitwear through to broad fibres which give carpets their strength and character. This means that wool gives designers endless potential for their creations - from delicate fabrics to rugged outdoor wear.
• Static Resistance	Because wool naturally absorbs moisture from the air, its tendency to collect static electricity is reduced.
• Noise Insulation	As mentioned above, wool is a wonderful insulator against heat and cold, but it is also a very good insulator against noise. It absorbs sound and reduces noise level considerably. For this reason wool wallpaper is often used in offices, restaurants, airport terminals, etc. Wool is also an ideal material for use in such places as concert halls to attain the best acoustics possible.

Table 1.2 – (continued)

• Dirt Resistance	Wool resists dirt, retains its appearance, and stays cleaner longer. Its ability to absorb moisture prevents a build-up of static electricity and therefore wool does not attract lint and dust from the air. Furthermore, the crimp in the wool fibre and the scales on the outside of the fibre assist in keeping dirt from penetrating the surface. These same qualities also make it easier to clean.
• Fashion	Leading designers throughout the world prefer to use wool - it comes in a wide choice of textures, weaves and weights, and is suitable for any style required.
• Dyeing	Wool dyes very easily and the range of colours is limitless. The scales on the surface of the wool fibre tend to diffuse light giving less reflection and a softer colour. Because proteins in the core of the fibre are reactive, they can absorb and combine with a wide variety of dyes. This means that the wool holds its colour well as the dye becomes part of the fibre.
• Comfort	Wool is comfortable to wear because its elasticity makes garments fit well and yield to body movement. It absorbs moisture, allows your body to breathe, and yet never feels damp and clammy. No other fabric serves so well under such a variety of conditions, nor combines so many natural properties.

1.1.2 THE MORPHOLOGICAL STRUCTURE OF WOOL

The textile industry uses substantial quantities of fibres obtained from various animal sources, of these sheep wool is the most important commercially (Rippon 1992). Early sheep were probably domesticated not for their wool, but rather as a source of food and skins. The most important breed for producing premium fine wools is the merino, which originated in Spain during the Middle Ages. This breed was so highly valued that their export was forbidden until the eighteenth century, when they were introduced into other countries. The most noteworthy of

which was Australia, where the breed has been developed to produce highly prized wool with exceptional fineness, length, colour, lustre and crimp.

A merino wool fibre, viewed under the scanning electron microscope is shown in Figure 1.1. Raw wool contains 25-70% by mass of impurities. These consist of wool grease, perspiration products (suint), dirt and vegetable matter such as burrs and seeds (Rippon 1992; Garner 1967; Pearson *et al*. 2004; Glaser 1996). These impurities are removed by specific processes (scouring and/or carbonizing) that will be further explained ahead in section 1.2 (Pearson *et al*. 2004; Lewis 1992). The wool discussed in this chapter is the fibrous material from which the surface contaminants, described above, have been removed.

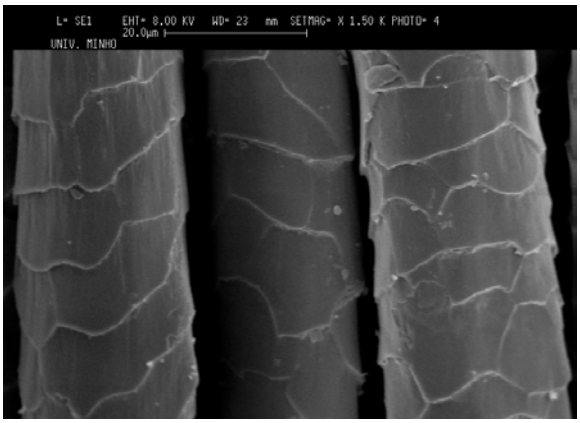


Figure 1.1 – Scanning electron micrograph of clean merino wool fibres.

Wool is an extremely complex protein, evolved over millions of years for the protection of warm-blooded animals in a great variety of climates and conditions. Wool is produced in the fibre follicle in the skin of the sheep. Because of the multitude of variations possible in, for example, the diet, breed and health of the sheep, as well as the climate, wool fibres vary greatly both in their physical properties, such as diameter, length and crimp, as in their chemical composition (Pailthorpe 1992).

Wool consists principally of one member of a group of proteins called keratins (Hughes *et al*. 2001; Fiadeiro *et al*. 2000; Hogg *et al*. 1994). Keratin fibres are not chemically homogeneous; they consist of a complex mixture of widely different polypeptides (Rippon 1992). Despite the classification of wool as a keratin, clean wool in fact contains only approximately 82% of the keratinous proteins, which are characterized by a high concentration of cystine. Approximately 17% of wool is composed of proteins which have been termed nonkeratinous, because of their relatively low cystine content (Rippon 1992). The wool fibre also contains approximately 1% by mass of non proteinaceous material; this consists mainly of waxy lipids plus a small amount of polysaccharide material. The nonkeratinous proteins and lipids are not uniformly distributed throughout the fibre but are concentrated in specific regions of the structure (Rippon 1992). Their location and their importance in determining the behaviour of wool are discussed later in this section.

A significant proportion of the polypeptide chains in wool are believed to be in the form of an α–helix, this ordered arrangement being responsible for the characteristic X-ray diffraction pattern of α–keratin (Hogg *et al*. 1994). The individual peptide chains in wool are held together by various types of covalent crosslinks and noncovalent interactions (Figure 1.2). In addition to their occurrence between separate polypeptide chains (inter-chain), these bonds can also occur between different parts of the same chain (intra-chain). With respect to the properties and performance of wool, however, inter-chains bonds are the more important of the two types (Rippon 1992).

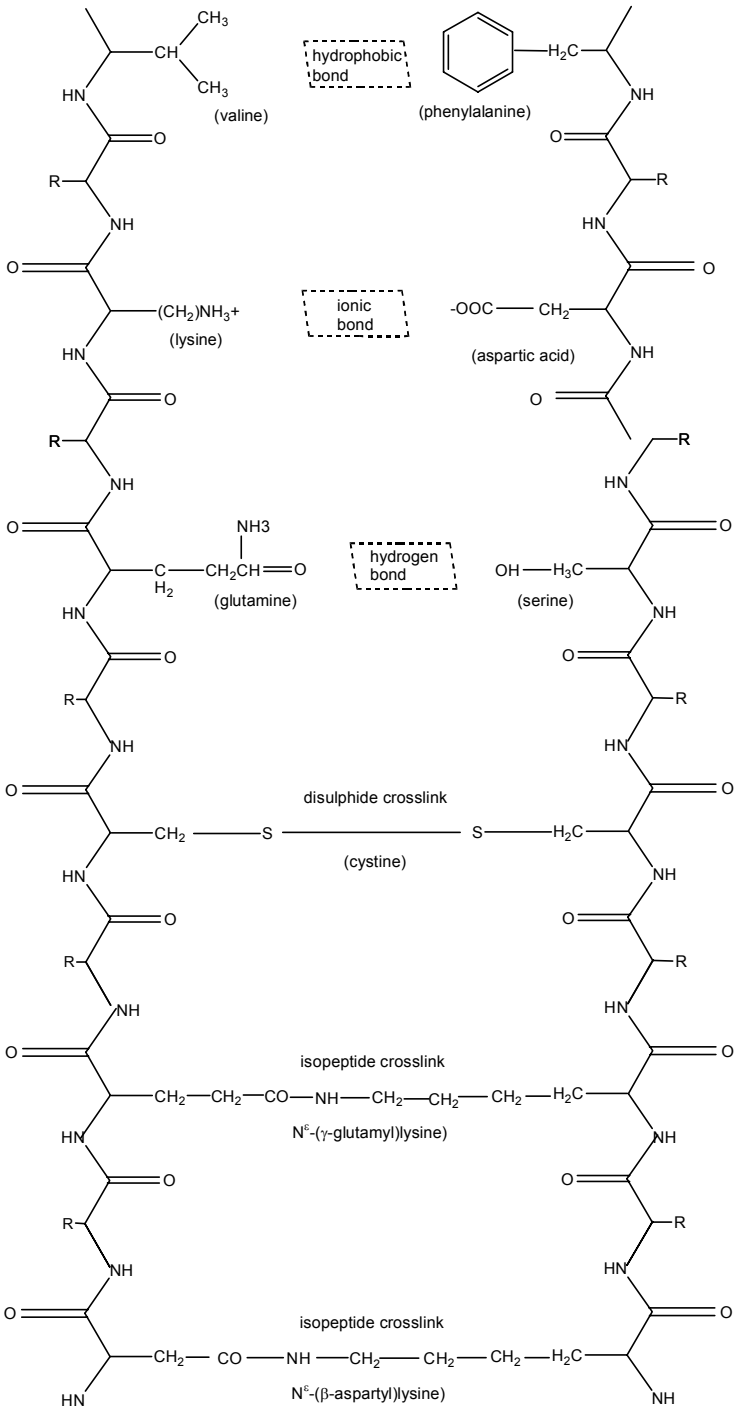


Figure 1.2 – Possible bonds between different wool protein chains, according to Rippon (1992).

Thus, wool is a complex natural fibre composed mainly of proteins (97%) and lipids (1%), with a heterogeneous morphological structure (Heine and Höcher 1995). Wool fibres have approximately the form of elliptical cylinders, with average diameters ranging from 15 μm to 50 μm and lengths determined by the rate of growth of the wool and the frequency of shearing (Makinson 1979). Wool and other keratin fibres consist of two major morphological parts: the cuticle layer (usually referred as scale layer of wool) which is composed of overlapping cells that surround the cortex (inner part of the fibre). The cortex comprises spindle-shaped cortex cells that are separated from each other by a cell-membrane complex (Figure 1.3), which consists of non-keratinous proteins and lipids (Feughelman 1997; Rippon 1992; Makinson 1979; Plowman 2003; Negri *et al*. 1993).

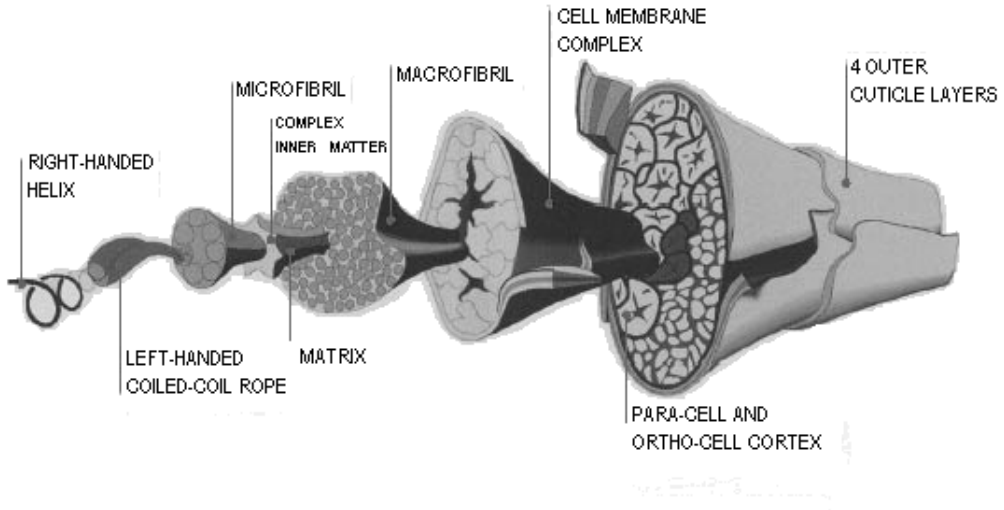


Figure 1.3 – Cross-section diagram of a merino wool fibre showing the structure at progressive magnifications, according to Feughelman (1997).

The cuticle cells are laminar, rectangular structures which form a sheath of overlapping scales enveloping the cortex (Speakman 1985; Naik 1994; Negri *et al.* 1993). They comprise 10% of the total weight of the wool fibre (Naik and Speakman 1993). The

cuticle cells are composed of three distinct layers, shown in Figure 1.4. The outermost layer is the outer resistant surface membrane (epicuticle); the next layer from the surface of the cells is called the exocuticle, which is subdivided into two main layers (A and B-layers) that differ mainly in the cystine content. Finally the endocuticle is the cuticular layer nearest to the cortex (Naik 1994; Heine and Höcker 1995; Rippon 1992; Feughleman 1997).

The substructure of the cuticle cells is directly relevant to felting, friction and shrinkproofing processes. The epicuticle, which constitutes about 0.25 percent of the total mass of the fibre, is very inert chemically, being resistant to acids, oxidising and reducing agents, enzymes, and alkalis (Makinson 1979; Negri *et al*. 1993). This membrane does not form a continuous sheet over the whole fibre, but covers the outer surface of each cuticle cell (Naik 1994). The epicuticle membrane is raised in the form of characteristic bubbles or sacs (Allworden bubbles) when the fibre is immersed in aqueous chlorine solutions (Makinson 1979; Rippon 1992). The epicuticle is known for its hydrophobicity, probably due to the lipid component bound to the membrane (Negri *et al*. 1993). The resistance of the surface membrane is thought to be due to the naturally occurring covalent isopeptide crosslinks as well as to covalent attached lipid, predominantly 18-methyleicosanoic acid (Naik 1994; Negri *et al*. 1993; Brack *et al*. 1999; Swift and Smith 2001; Heine and Höcker 1995). This fatty acid is covalently bound to the protein matrix via cysteine residues, forming a layer that can be removed by treatment with alcoholic alkaline or chlorine solutions in order to enhance many textile properties such as wetability, dye uptake and polymer adhesion (Negri *et al*. 1993; Brack *et al*. 1999).

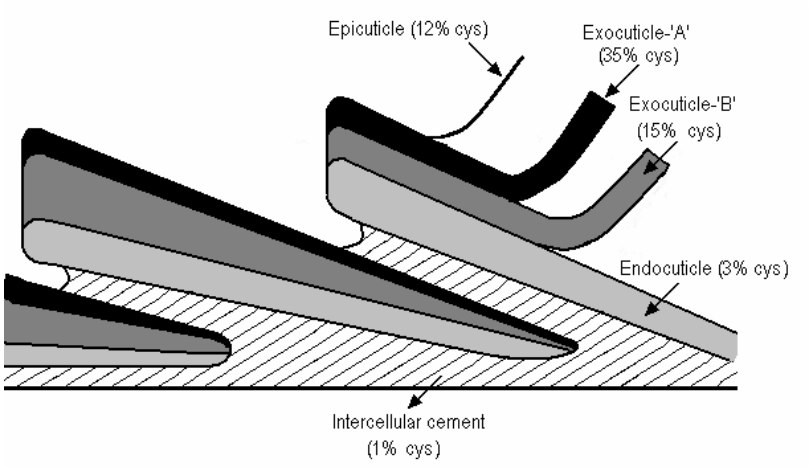


Figure 1.4 – Schematic scale structure of the cuticle showing the major components (based on Rippon (1992).

Thirty-five percent of the exocuticle A-layer is made by cystine residues, and in addition to normal peptide bonds, the cuticle is crosslinked by isodipeptide bonds (ε-(γ-glutamyl)lysine) (Naik 1994; Rippon 1992; Heine and Höcker 1995). The A and B-layers are both resistant to boiling in diluted hydrochloric acid and to trypsin digestion; however they can be solubilised by trypsin treatment after oxidation or reduction. The endocuticle is preferentially attacked by proteolytic enzymes, and readily degraded in diluted boiling hydrochloric acid (Naik 1994; Sawada and Ueda 2001). Therefore, as pointed out by several authors, wool cuticle forms a diffusion barrier to chemicals and other treatment agents (Naik 1994; Schäfer 1994; Nolte *et al*. 1996). This diffusion barrier (to dye molecules, for example) is mostly due to the hydrophobic character of the exocuticle A-layer, caused by the large amount of disulphide crosslinks and the bound lipid material. Consequently, the fibre pre-treatment processes modify mainly the composition and morphology of the wool surface (Brack *et al*. 1999; Millington 1998; Pascual and Julia 2001).

1.1.3 FELTING AND SHRINKAGE

One of the intrinsic properties of wool, that is peculiar to wool only, is its tendency to felting and shrinkage. Under certain conditions, such as moisture, heat and mechanical agitation, wool shrinks, basically due to its morphological and scale structure. There are two kinds of shrinkage: relaxation shrinkage and felting shrinkage.

Relaxation shrinkage describes the shrinkage which appears during production when fabrics are subjected to more or less strong mechanical tensions in warp or in weft direction. In finished garments these tensions can still be present either completely or partially. If such a garment gets wet either during wearing or washing, these tensions are loosened and the garment shrinks. The antifelt finishing does not remove relaxation shrinkage. Therefore, to obtain a lower relaxation shrinkage the garment must be processed with as less tension as possible or it must be relaxed by corresponding finishing processes.

Felting shrinkage describes the shrinkage of garments due to the felting of wool fibres. Legends state that the felting of wool was discovered by a mediaeval saint who packed wool or fur into his shoes to ease his blistered feet and subsequently found that it had matted into a fabric. Only keratin fibres, grown on animals from their skin, can be induced to felt. This is because a directional surface structure is provided by the scales (the cuticle scales are arranged towards the fibre tip) which occur on all animal fibres but are not present on vegetable or man-made fibres (Makinson 1979). Hence, the friction of a wool fibre in the scale direction is lower than the friction against the scale direction. There are different theories concerning the origin of wool felting (Heine 2002). The hydrophobic character and the scaly structure of the wool surface are the main factors causing

the differential frictional effect (DFE) resulting all fibres to move to their root end when mechanical action (such as moisture, heat, and pressure) is applied in the wet state (Makinson 1979; Chi-wai *et al*. 2004; Höcker 2002). The felting changes not only the garment dimensions but its look as well. The woven or knitted structure becomes less visible, and the garment becomes thicker and less elastic.

The word shrinkproofing means, in practice, treating textiles to reduce felting shrinkage; it does not as a rule imply the prevention of relaxation shrinkage. Thus, a better term is proposed for this type of finishing process: antifelt-finishing (Makinson 1979). The commercially implemented antifelt-finishing processes will be described briefly in section 1.2. These shrinkproofing processes aim at the modification of the fibre surface either by oxidative or reductive methods and/or by the application of a polymer resin onto the surface.

1.2 Conventional Finishing Processes for Wool Fibre

A variety of processes are available to improve the appearance, handle, performance and durability of the wool fabrics. Before the more specialised finishing processes are applied, fabrics usually require cleaning (scouring) to remove warp sizing, oils, other additives and dirt. Processes such as bleaching and dyeing are known as wet-finishing processes, since the fabric is exposed to bleaches or dyes in aqueous solutions. Treatment of fabric with particular resin systems can improve crease and shape resistance. These are examples of chemical finishing processes, which depend upon the ultimate use of the textile. (Glaser 1996). Some of the finishing processes in which enzymes can be employed

will be described later (section 1.4). In common with almost all other manufacturing activities, wool processing has the potential to cause environmental damage that should be eliminated or minimized for wool to maintain its naturalness. Pollution is a concern in several areas of wool finishing. In a study performed by the International Wool Secretariat (IWS), four high priority areas were identified (Shaw 1996):

- Pesticide residues in wastewater from chemicals applied to sheep.
- Discharge of mothproofing agents from wool carpet manufacture.
- Emissions of halo-organics from wool shrinkproofing.
- Chromium releases from chrome dyeing operations.

The finishing processes that may be carried out on wool prior to commercialization are discussed bellow. Such processes include scouring, carbonizing, bleaching, dyeing, antimicrobial finishing and shrinkproofing.

Scouring

Washing, also denominated as scouring, is the first process that raw wool goes through, and its purpose is to remove the dirt, grease, and other impurities. As already referred, wool fleeces usually contain less than 50% of clean fibre, being heavily contaminated by wool wax, skin flakes, suint, sand, dirt and vegetable matter (Araújo and Melo e Castro 1984; Dominguez *et al*. 2003). To achieve satisfactory wool products, these contaminants need to be efficiently removed by scouring with sodium carbonate and non-ionic surfactants (Tomasino 1992; Dominguez *et al*. 2003). The pollution load from a wool scouring mill can be equivalent to the normal discharge from a small town, and steps must therefore be taken to recover at least some of the contaminants before discharge (Lewis 1992; Jones and Westmoreland

1999). The wool emerges at the end of this process about 30 percent lighter than its original weight. The grease that is removed (lanolin) is considered a valuable by-product. Raw wool contains 10 to 25 percent grease, or lanolin, which is recovered during the scouring process (Glaser 1996). Lanolin consists of a highly complex mixture of esters, alcohols, and fatty acids and is used in adhesive tape, printing inks, motor oils, and auto lubrification. It can also be purified for use in the manufacture of many cosmetics and pharmaceuticals (Glaser 1996; Phillips 2004; López-Mesas *et al*. 2000).

Carbonising

Scouring does not always eliminate vegetable matter such as burrs and seeds. Heavily contaminated wool must go through a process known as carbonising. If burrs are not removed at this stage they can cling to the wool fibres and not be noticed until the process is complete. The process of carbonising is the treatment of the vegetable matter with sulphuric acid and heat. The wool is steeped in the acid solution which causes the burrs to break up. The wool is then subjected to heat which converts the disintegrated material into carbon. The burrs are then finally removed, firstly by crushing and then shaken out of the wool by a machine rotating at high speed (Araújo and Melo e Castro 1984). Any remaining impurities are thus blown out of the fibre. Following carbonising, the wool should be rinsed and neutralised by a wet process. Such neutralisation should be carried out immediately after baking, otherwise fibre damage will occur during storage of the wool in such an acidic state (Araújo and Melo e Castro 1984; Lewis 1992; Tomasino 1992).

Bleaching

Only a small percentage of the total world production of wool is bleached (Araújo and Melo e Castro 1984; Lewis 1992) and this operation is only performed when wool is intended to be white dyed or light dyed, like for baby clothes. Hydrogen peroxide and peroxy compounds damage wool fibres, due to progressive oxidation of disulfide bonds ultimately forming cysteic acid (Gacen and Cayuela 2000).

Dyeing

Dyeing operations are used at various stages of production to add colour and sophistication to textiles and increase product value. Wool textiles are dyed using a wide range of dyestuffs, techniques, and equipment (EPA 1997). Until fairly recently, most of the dyes used on wool were acid dyes. Nowadays, acid, chrome, metal-complex and reactive dyes may all be used for the dyeing of wool (Pailthorpe 1992).

Antimicrobial Finishing

Textiles are an excellent medium for the growth of microorganisms when the basic requirements such as nutrients, moisture, oxygen, and appropriate temperature are present. Natural fibres are more susceptible to microbial attack than synthetic fibres, and unlike any other textile fibres, wool and other protein fibres are subject to attack by the larvae of certain moths (*Lepidoptera* sp.) and beetles (*Coleoptera* sp.). In the carpet industry, the antimicrobial and/or mothproofing of wool fabric is an important finishing step. A durable antimicrobial

finish is a potentially effective means of controlling microorganisms on a textile. Various chemicals have been applied to wool to control microbial and larval attack (Purwar and Joshi 2004; Han and Yang 2005). Especially in the last decades, however, considerable environmental restrictions have been placed on the type of agent which may be employed. Magnesium hydroperoxide and related compounds, and chitin and chitosan based antimicrobial agents are the new generation of environmentally friendly antimicrobial agents (Purwar and Joshi 2004). Non-toxic natural dyes have also been tested on the antimicrobial activity of wool with good results (Han and Yang 2005).

Shrink-resist Treatments

Wool is a relatively expensive fibre when compared with other natural and synthetic fibres and its position as a high quality, luxury fibre permits relatively costly processing and finishing treatments to be carried out, increasing the value of wool textiles. One of the intrinsic properties of wool and other animal fibres, as previously stated, is their tendency to felt and shrink thus limiting the use of untreated wool materials as machine washable textiles. The shrinkage behaviour of wool can be regulated to a greater or smaller degree by various chemical means; however, choices are more limited if it is desired to achieve the high performance demanded by consumers, particularly with the increasing tendency towards tumble dryability properties as part of the consumer trend to “easy-care” properties. There are various successful commercial shrink-resist processes available for textile industries that have been developed decades ago. The processes which have been developed so far can be combined in 3 groups:

a. Subtractive processes – The oldest type of shrinkproofing treatment involves

chemical attack on the cuticle of the fibres. Such treatments are commonly called degradative processes. The earliest degradative treatment was chlorination, introduced as a shrinkproofing treatment during the latter half of the nineteenth century (Makinson 1979). The principal mode of action of degradative antifelting treatments is that they make the cuticle cells softer in water than those of untreated fibres. This softening is the result of oxidation and scission of the numerous disulfide bonds in the exocuticle of the wool (Makinson 1979). This softening, which results from an increase in the number of charged and/or soluble molecules contained inside the cell membrane, causes a reduction in the directional frictional effect (Makinson 1979).

b. Additive processes – The second class of shrinkproofing treatments consists of those in which a polymer is added to the wool; they are also called additive treatments. The polymer is deposited on the surface of the fibres, and it can be applied to the fabric/yarn or to the wool tops. The polymers which are effective only when applied to fabric or yarn act by sticking the fibres together at the points where they touch or come close together. This prevents relative movement between the fibres of the magnitude necessary for felting. The polymers that are applied to tops require a pre-treatment which is, in practice, a mild chlorination. Thus, this treatment is the combination of these two processes.

c. Combination of subtractive and additive processes (e.g. chlorine-Hercosett process) - Nowadays the combination of subtractive and additive processes has the highest importance in commercial processes. To date the most successful process for producing truly machine-washable wool is the chlorine-Hercosett process. This extremely effective process consists of a

strongly acid chlorination step followed by a dechlorination step (subtractive process) with a subsequent application of a cationic polymer (Hercosett: polyamide-epichlorohydrin resin) (additive process). The chlorination results in the oxidation of cystine residues to cysteic acid residues in the surface of the fiber and allows the cationic polymer to spread and adhere to wool surface (Lewis 1992; Heine 2002).

The chlorine-Hercosett process has been dominant in the industry for 30 years, and still about 75% by weight of the world's treated wool is processed by this route in one of its forms (Holme 2003). With respect to efficiency and cost the chlorine-Hercosett process offers tremendous advantages which are hard to overcome even with great effort: excellent antifelt effect, purposive modification of the wool surface, low damage and low weight loss. From today's modern point of view, however, the chlorine-Hercosett process shows a number of drawbacks which make the search for an ecologically clean alternative worthwhile: limited durability, poor handle, yellowing of wool, difficulties in dyeing and the most important today, environmental impact (release of absorbable organic halogens-AOX to the effluents) (Heine 2002; Julia *et al*. 2000; Schlink and Greeff 2001).

Therefore, because of the new environmental directives, the development of clean technologies such as enzymatic finishing processes is a priority. The biotechnology applied to textile industry, by the use of enzymes, has already contributed to a reduction of energy costs and also to the reduction of pollutant emissions into the environment (Feitkenhauer and Meyer 2001).

Before introducing some of the enzymatic finishing processes already implemented in the textile industry (section 1.4), a brief description of enzymes

characteristics and their properties will be given in the following section.

1.3 CHARACTERISTICS AND PROPERTIES OF ENZYMES

Most of the reactions in living organisms are catalysed by protein molecules called enzymes. Enzymes can rightly be called the catalytic machinery of living systems and have played an important role in many aspects of life since the dawn of times. In fact they are vitally important to the existence of life itself. Enzymes are nature's catalysts (i.e. they speed up the rates of reactions without themselves undergoing any permanent change). Civilizations have used enzymes for thousands of years without understanding what they were or how they worked. Over the past several generations, science has unlocked the mystery of enzymes and has applied this knowledge to make better use of these amazing substances in an ever-growing number of applications. Today, nearly all commercially prepared foods contain at least one ingredient that has been made with enzymes. Enzymes also play a significant role in non-food applications. Industrial enzymes are used in laundry and dishwashing detergents, stonewashing jeans, pulp and paper manufacture, leather dehairing and tanning and desizing of textiles.

Some enzymes are still extracted from animal or plant tissues. Plant derived commercial enzymes include the proteolytic enzymes papain, bromelain and ficin and some other special enzymes like lipoxygenase from soybeans. Animal derived enzymes include proteinases like pepsin and rennin. Most of the enzymes are, however, produced by microorganisms in submerged cultures in fermentors.

Criteria used in the selection of an industrial enzyme include specificity,

reaction rate, optimum pH and temperature, stability, effect of inhibitors and affinity to substrates. Enzymes used in industrial applications must usually be tolerant against various heavy metals and have no need for cofactors.

The simplest way to use and apply enzymes to practical processes is to add them into a process stream where they catalyse the desired reaction and are gradually inactivated during the process. In these applications the price of the enzymes must be low to make their use economical. An alternative way to use enzymes is to immobilise them so that they can be reused. Many different methods for enzyme immobilization based on chemical reaction, entrapment, specific binding or adsorption have been developed.

Therefore, enzymes play crucial roles in producing the food we eat, the clothes we wear, even in producing fuel for our automobiles. Enzymes are also important in reducing energy, water, raw materials consumption and environmental pollution (by generating less waste and fewer environmental pollutants). In this section, a brief overview of the characteristics and properties of enzymes will be given.

1.3.1 NOMENCLATURE AND CLASSIFICATION OF ENZYMES

Presently more than 3000 different enzymes have been isolated, mainly from mesophilic organisms, and characterized (Kumar and Takagi 1999; Sharma *et al*. 2001). Only a limited number of all the known enzymes are commercially available and even a smaller amount is used in large quantities. At least 75% of all industrial enzymes are hydrolytic in action (Sharma *et al*. 2001). Protein-degrading enzymes

dominate the market, accounting for approximately 40% of all enzyme sales (Godfrey and West 1996; Sharma *et al*. 2001; Gupta *et al*. 2002). Proteases have found new applications but their use in detergents is still the major market.

Enzymes are categorized according to the compounds they act upon. As in the development of organic chemistry, many enzymes were given “trivial” names before any attempt was made to create a system of nomenclature. Some of the most common include: proteases which break down proteins, cellulases which break down cellulose, lipases which split fats (lipids) into glycerol and fatty acids, and amylases which break down starch into simple sugars. The present-day accepted nomenclature of enzymes is that recommended by the Enzyme Commission (EC), which was set up in 1955 (IUB 1992; Price and Stevens 1999; Fornelli 1995). The six major types of enzyme-catalysed reactions are:

1. oxidation-reduction reactions, catalysed by *Oxidoreductases*;
2. group transfer reactions, catalysed by *Transferases*;
3. hydrolytic reactions, catalysed by *Hydrolases*;
4. elimination reactions in which a double bound is formed, catalysed by *Lyases*;
5. isomerization reactions, catalysed by *Isomerases*;
6. reactions in which two molecules are joined at the expense of an energy source (usually ATP), catalysed by *Ligases*.

1.3.2 Properties of Enzymes as Catalysts

Enzymes are natural protein molecules that act as highly efficient catalysts

in biochemical reactions, i.e., they help a chemical reaction take place quickly and efficiently. This catalytic capability is what makes enzymes unique. Enzymes not only work efficiently and rapidly, but they are also biodegradable.

The use of enzymes frequently results in many benefits that cannot be obtained with traditional chemical treatments (Kragl 1996; Powell 1990). These often include higher product quality and lower manufacturing cost, less waste and reduced energy consumption (Silva and Roberto 2001; Bickerstaff 1997; Sarkar and Etters 1999). The traditional chemical treatments are generally non-specific, not always easily controlled, and may create harsh conditions. Often they produce undesirable side effects and/or waste disposal problems. The degree to which a desired technical effect is achieved by an enzyme can be controlled through various means, such as dosage, temperature, and time. Because enzymes are catalysts, the amount added to accomplish a reaction is relatively small.

With environment and cost issues surrounding conventional chemical processes being subjected to considerable scrutiny, biotechnology is gaining ground rapidly due to the various advantages that it offers over conventional technologies. Industrial enzymes represent the heart of biotechnology processes. According to a recent released report from Business Communications Company, Inc. (BCC 2004) the global market for industrial enzymes was estimated at US $2 billion in 2004. Volume growth of industrial enzymes is between 4% and 5% of the Average Annual Growth Rate (AAGR), which is accompanied by decreasing prices, due to the increase in the number of smaller players competing in the market. The industrial enzyme market is divided into three application segments: technical enzymes, food enzymes and animal feed enzymes. The following chart shows the global enzyme markets by application sectors, through 2009.

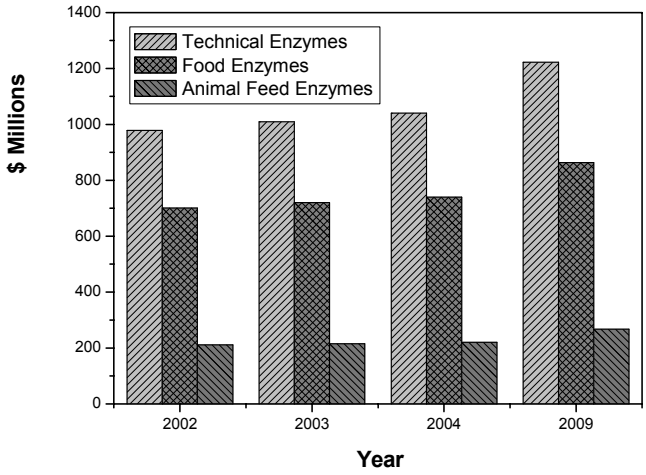


Figure 1.5 - Global enzyme markets by application sectors, through 2009 ($ Millions), according to BCC (2004).

Textile enzymes are the third most significant segment of the market of industrial enzymes, as can be depicted from Figure 6 (BCC 1998). The major enzymes in this category are enzymes for processing cotton and cellulosic textiles, followed by enzymes for processing leather and fur. The enzyme market for the treatment of silk and wool is minor (BCC 1998).

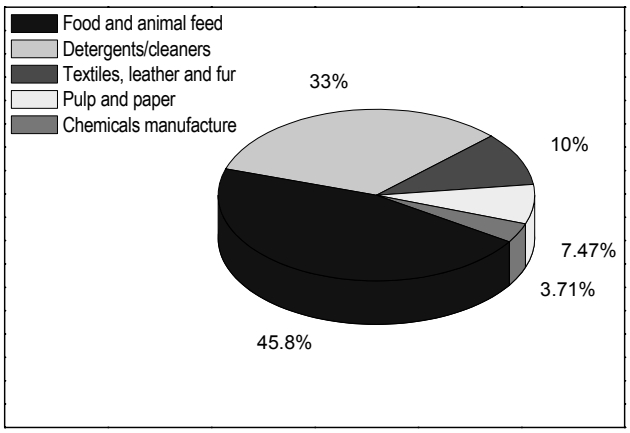


Figure 1.6 – Distribution of industrial enzymes: worldwide market forecast in 2002 ($ million), according to BCC (1998).

1.3.3 PROTEOLYTIC ENZYMES

Proteases have been used in food processing for centuries, like rennet obtained from calves' stomachs used traditionally in the production of cheese and papain from the leaves and unripe fruits of the pawpaw used to tenderize meats (Chaplin and Bucke 1990). Proteolytic enzymes catalyse the hydrolysis of certain peptide bonds in protein molecules, as already mentioned in the previous section. The general reaction can be illustrated by:

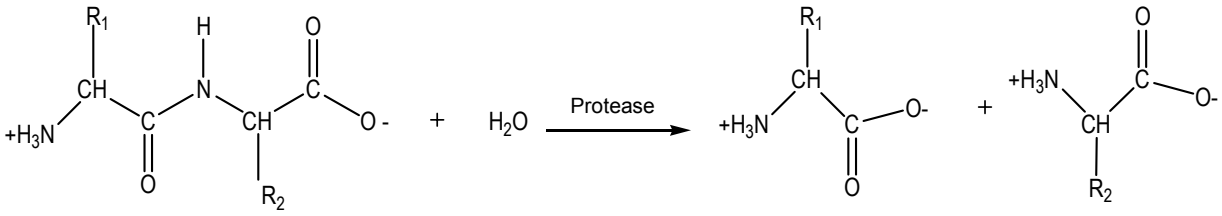


Figure 1.7 – Schematic representation of the cleavage of a peptide bond by a protease.

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology (Gupta *et al*. 2002). Today, proteases account for more than 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery (Godfrey and West 1996; Gupta *et al*. 2002; Sharma *et al*. 2001; Kumar and Takagi 1999; Rao *et al*. 1998; Banerjee *et al*. 1999; Singh *et al*. 2001). However, until today, the largest share of the enzyme market has been held by alkaline proteases and different companies worldwide have successfully launched several products based on these, in the past few years (Gupta *et al*. 2002).

Probably the largest application of alkaline proteases is as additives for detergents, where they help in removing protein based stains from clothing (Gupta *et al*., 2002; Kumar and Takagi 1999). In textile industry proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fibre to achieve improved lustre and softness (Freddi *et al*. 2003). Protease treatments can modify the surface of wool and silk fibres to provide new and unique finishes. Research has been carried out on the application of proteases to prevent wool felting (Silva and Cavaco-Paulo 2003; Cortez *et al*. 2004; Heine and Höcker 1995; Heine 2002). The bio-industrial viewpoints of microbial alkaline proteases have been reviewed (Gupta *et al*. 2002; Kumar and Takagi 1999).

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms (Rao *et al*. 1998). In earlier days, proteases were classified according to molecular size, charge or substrate specificity. A more rational system is now based on a comparison of active sites, mechanism of action and three-dimensional structure (Neurath 1996; Rao *et al*. 1998).

Proteases attack proteins via two modes, yielding different products (Hsieh and Cram 1999; Romero *et al*. 2001). Exopeptidases act cleaving off single amino acids from either end of the peptide chain. Exoproteases are specific according to which end of the protein chain they attack, either carboxypeptidases if they attack the end with a free carboxylic acid (C-terminus) or aminopeptidases if they attack the free amino end group (N-terminus). Endopeptidases or proteinases attack peptide bonds in the interior of the peptide chain, yielding smaller polypeptides and peptides. The endoproteases are classified according to the mechanism of their active site. Four mechanistic classes: serine and cysteine proteases (which form

covalent enzyme complexes) and aspartic and metallo-proteases (which do not form covalent enzyme complexes) are recognized by the International Union of Biochemistry (IUB 1992), and within these classes, six families of proteases are distinguished to date. Each family has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site (see Table 4) (Neurath 1996). Members of each family are believed to have descended from a common ancestor by divergent evolution.

Table 1.3 - Families of proteolytic enzymes, according to Neurath (1996)

Family[a]	*Representative protease(s)*	*Characteristic active site residues*[b]
Serine proteases I	**Chymotrypsin** Trypsin Elastase Pancreatic kallikrein	Asp^{102}, Ser^{195}, His^{57}
Serine proteases II	**Subtilisin**	Asp^{32}, Ser^{221}, His^{64}
Cysteine proteases	**Papain** Actinidin	Cys^{25}, His^{159}, Asp^{158}
Aspartic proteases	**Penicillopepsin** Rhizopus chineses and Endothia parasitica, acid proteases Renin	Asp^{33}, Asp^{213}
Metallo-proteases I	**Bovine carboxypeptidase A**	Zn, Glu^{270}, Try^{248}
Metallo-proteases II	**Thermolysin**	Zn, Glu^{143}, His^{231}

[a] This table includes only enzymes of known amino acid sequence and three dimensional structure.
[b] The number of residues corresponds to the amino acid sequence of the enzyme listed in bold column 2.

The serine peptidases are the most thoroughly studied class of enzymes in the protease field, and perhaps in all of enzymology (Dunn 1996), being characterized by the presence of a serine group in their active site (Rao *et al*. 1998).

They are numerous and widespread, suggesting that they are vital to the organisms. The serine proteases include two distinct families: the mammalian serine proteases (for example chymotrypsin and trypsin) and the bacterial serine proteases (for example subtilisin). They differ from each other in amino acid sequence and three-dimensional structure, despite a common active site geometry and enzymatic mechanism. Analogously, the metallo-proteases include two families: the mammalian and bacterial metallo-proteases.

One of the most extensively studied families of serine proteases is the subtilisin family. It consists of primarily prokaryotic proteases, such as subtilisin BPN´ from *Bacillus amyloliquefaceins*, but subtilisins have also been discovered in organisms such as fungi and higher eukaryotes.

In this research, a subtilisin (EC 3.4.21.62) was used. This protease promotes the hydrolysis of proteins with broad specificity for peptide bonds. It also hydrolyses peptide amides. Subtilisin is a serine endopeptidase, and it contains no cysteine residues. This enzyme, which has no disulfide bonds, consists of a single polypeptide chain of about 275 aminoacids residues and possesses the “catalytic triad” of Asp, His and Ser residues that is conserved among serine proteases as the hallmark of its active site (Takagi and Takahashi 2003). Species variants include subtilisin BPN' (also subtilisin B, subtilisin Novo, bacterial proteinase Novo) and subtilisin Carlsberg (subtilisin A, alcalase Novo). Similar enzymes are produced by various *Bacillus* strains (PDB).

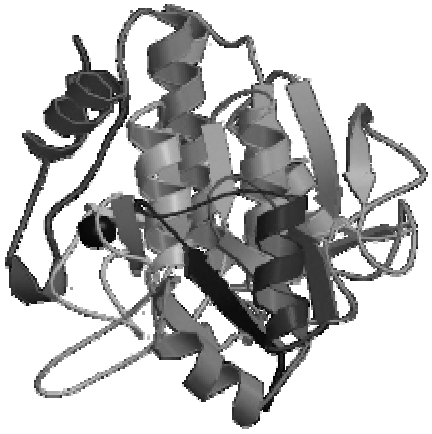


Figure 1.8 – Ribbon drawings of X-ray structure of a subtilisin from *Bacillus* sp. (from PDB, entry 1SCN). Arrows denote beta-sheets and spirals denote helices.

1.3.4 ENZYME IMMOBILIZATION BY COVALENT COUPLING

An important factor determining the use of enzymes in a technological process is their cost (Chaplin and Bucke 1990; Swaisgood 1991; Ciardelli and Ciabatti 2002). Immobilization of enzymes often incurs an additional expense and is only undertaken if there is a solid economic or process advantage in the use of the immobilized, rather than free (soluble) enzymes. The interest in the use of immobilized enzymes in industry is based on the potential advantages they confer over their soluble counterparts, including increased stability to temperature, pH and organic solvents; recovery and reuse of the enzyme; and, in the case of proteases, removal or reduction of autolysis or denaturation (Park *et al*. 2002; Kumar and Takagi 1999).

The most important benefit derived from immobilization is the easy separation of the enzyme from the products of the catalysed reaction. This

prevents the enzyme from contaminating the product, minimising downstream processing costs and possible effluent handling problems. It also allows continuous processes to be practicable, with a considerable saving in enzyme, labour and operating costs. Immobilization often affects the stability and activity of the enzyme, but conditions are usually available where these properties are hardly changed or even enhanced (Chaplin and Bucke 1990; Bickerstaff 1997; Worsfold 1995). However, insoluble immobilized enzymes are of little use when the substrate is also insoluble, due to steric hindrance and diffusional limitations (Chaplin and Bucke 1990; Kumar and Gupta 1998; Fujimura *et al*. 1987; Chen 1998).

Immobilized enzymes have been defined as enzymes that are physically confined or localized, with retention of their catalytic activity, and which can be used repeatedly and continuously (Worsfold 1995). There is a variety of methods by which enzymes can be immobilized, ranging from covalent chemical bonding to physical entrapment. However they can be broadly classified as follows (Powell 1990; Bickerstaff 1997; Bullock 1989, Kennedy and Roig 1995; Worsfold 1995; Carr and Bowers 1980):

1) Adsorption of the enzyme into a support material.
2) Covalent binding of the enzyme to a support material.
3) Entrapment by intermolecular cross-linking of enzyme molecules using multi-functional reagents.
4) Encapsulation by membrane confinement of the enzyme inside a water-insoluble polymer lattice or semi-permeable membrane.

Immobilization of biocatalysts has gained popularity over the past decades. Among the different methods of immobilization techniques considered (Figure 9), covalent coupling of the enzymes to matrices is an extensively researched technique due to its own merit and to the benefits in the repeated use of

biocatalysts in bioconversions and down-stream processing (Arasaratnam *et al*. 2000). This method of immobilization involves the formation of a covalent bond between the enzyme and the support material, or matrix (Bickerstaff 1997). The strength of binding is strong, and very little leakage of enzyme from the support occurs. The relative usefulness of various groups, found in enzymes, for covalent link formation depends upon their availability and reactivity (nucleophilicity), in addition to the stability of the covalent link, once formed. Lysine residues are found to be the most generally useful groups for covalent bonding of enzymes to insoluble supports due to their widespread surface exposure and high reactivity, especially in slightly alkaline solutions. They also appear to be only very rarely involved in the active sites of enzymes.

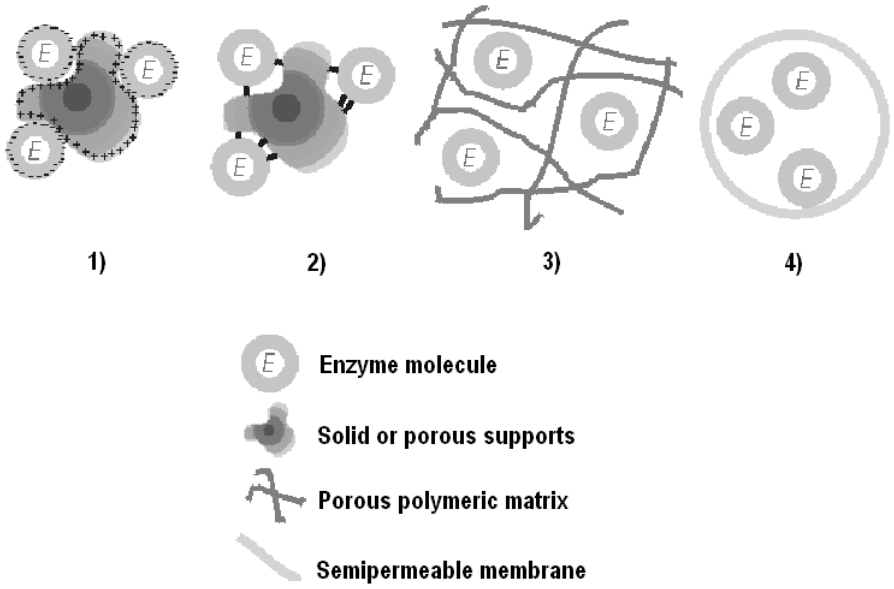


Figure 1.9 - Immobilised enzyme systems. (1) enzyme non-covalently adsorbed to an insoluble particle; (2) enzyme covalently attached to an insoluble particle; (3) enzyme entrapped within an insoluble particle by a cross-linked polymer and (4) enzyme confined within a semi-permeable membrane (Based on Worsfold 1995; Carr and Bowers 1980 and Chaplin and Bucke 1990).

Generally it is found that covalent immobilization is more effective than the other methods of immobilization in improving enzyme resistance to heat, chemical disruption and pH changes (Bickerstaff 1997). This method is also useful to avoid one of the major causes of inactivation of proteases which is the proteolytic self-degradation (autolysis). This can be avoided by covalently binding proteases onto supports to prevent them from attacking each other (Bickerstaff 1997; Stoner *et al*. 2004), although some loss of enzyme activity might occur.

Carbodiimides are very useful bifunctional reagents as they allow the coupling of amines to carboxylic acids. Careful control of reaction conditions and choice of carbodiimide allow a great degree of selectivity in this reaction. Glutaraldehyde is another bifunctional reagent which may be used to cross-link enzymes or link them to supports. This bifunctional aldehyde is relatively cheap, available in industrial quantities and has the additional advantage to be a biocide and sanitize the biocatalyst (Kragl 1996). There are other numerous methods available for the covalent attachment of enzymes (e.g. the attachment of tyrosine groups through diazo-linkages, and lysine groups through amide formation with acyl chlorides or anhydrides).

Proteases have been immobilized using a wide range of methods including deposition or precipitation into porous supports and covalent attachment to activated supports. Attachment of alkaline proteases to insoluble carriers (by either physical adsorption or covalent coupling) is the most prevalent method of immobilization. Various carriers employed for this purpose include porous glass (Wilson *et al*. 1994; Parrado and Bautista 1995), silica (Haensler *et al*. 1997; Ferreira *et al*. 2003), resins (Chae *et al*. 2000), nylon (Chellapandian and Sastry 1994), chitosan and chitin (Abdel-

Naby *et al*. 1998; Farag and Hassan 2004), polyester (Nouaimi *et al*. 2001) and vermiculite (Chellapandian 1998).

There are also reports of the immobilization of proteases into soluble matrices, like polyethylene glycol (Gaertner and Puigserver 1992) or soluble-insoluble polymers (Kumar and Gupta 1998; Fujimura *et al*. 1987; Sharma *et al*. 2003; Arasaratnam *et al*. 2000; Chen 1998). These polymers can easily be reversibly made soluble-insoluble by changing the physical conditions, such as pH and temperature, and by adding certain ions. Thus, if enzymes are attached to soluble-insoluble polymers, they present several advantages since they can be used as catalysts in their soluble form and then be recovered by precipitation for reuse, being easily separated from the products. Moreover, they overcome the problem of the non accessibility of the enzyme to macromolecular substrates. One of such supports with reversible solubility is Eudragit S-100, a random copolymer of methacrylic acid and methyl methacrylate. Enzymes coupled to Eudragit have been used for hydrolysis of macromolecular substrates, such as starch, cellulose, olive oil, xylan, and casein, among others (Dourado *et al*. 2002; Sardar *et al*. 1997; Charusheela and Arvind 2002; Rodrigues *et al*. 2002; Sardar *et al*. 2000; Cong *et al*. 1995; Taniguchi *et al*. 1989; Teotia *et al*. 2003).

The bioconjugates formed with Eudragit S-100 have several attractive features which favours its use as an immobilization matrix for enzymes/proteins. It is non-toxic (safe for use in food processing industries), water soluble (allowing its use for macromolecular substrates), recoverable from solution by pH alteration, economical, and commercially available (Kumar and Gupta 1998). According to Valuev *et al*. (1998), to prevent the decrease in the biological activity of immobilized proteins, it is necessary to carry out the immobilization process in two steps. The first step should be the activation of the polymer surface by carbodiimides, and the

second step should be the modification of these surfaces by enzymes. The coupling of the polymer to enzymes using water-soluble carbodiimides can be illustrated by the following scheme:

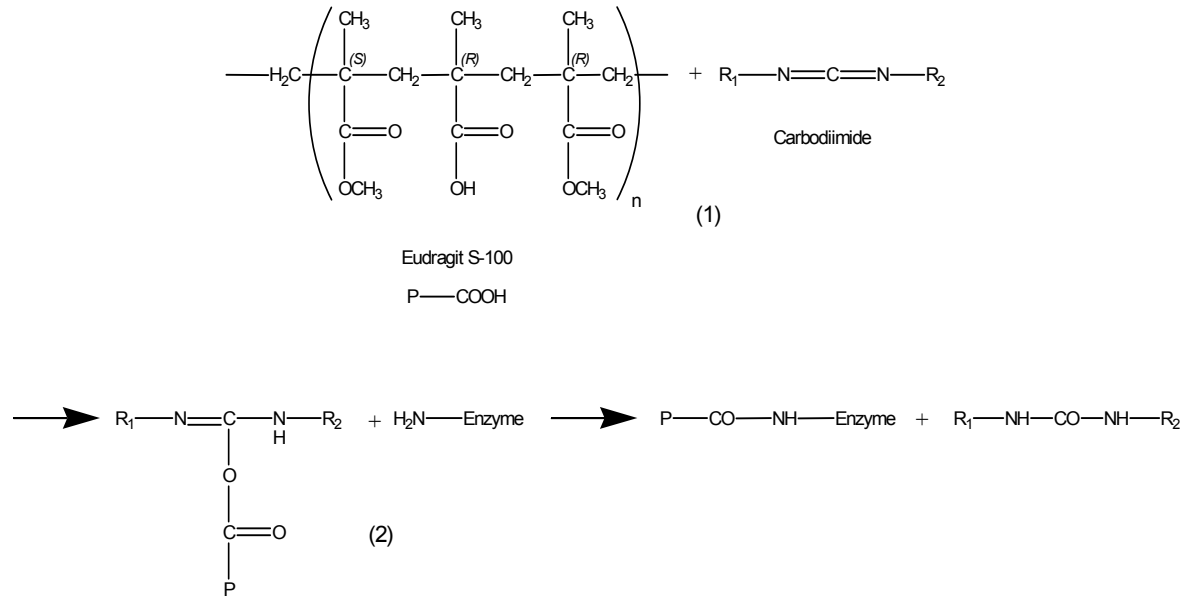


Figure 1.10 – Schematic representation of the different steps in the immobilization of enzymes to Eudragit S-100: (1) activation of the polymer with soluble carbodiimide and (2) coupling reaction with the enzyme.

1.4 ENZYMATIC FINISHING PROCESSES FOR WOOL

Textile processing requires the use of vast amounts of water (about 100 L of water by 1 kg of processed textile material, Rodrigues 2000), chemicals and energy, and therefore it has important effects on the quality of the environment in textile manufacturing regions (Cooper 1993). There are many strategies which a manufacturer might use for reducing potential sources of pollution (such as process modifications, reuse, recovery, and other control strategies). In most

cases, the results that can be obtained are site-specific, and each manufacturing process must be evaluated on its own merit as a separate situation (Cardamone and Marmer 1995). One control strategy that has given proof of efficiency in replacing pollutant processes in textile industry is, as already mentioned, the use of enzymes to replace the conventional chemical processes. Biotechnology processing was introduced into the textile industry in the beginning of the 20th century, being facilitated by the use of water solutions and relatively mild conditions in the textile processes (Sørup *et al*. 1998), and specifically aimed at minimising the environmental effects as well as improving product quality (reducing the damage caused to the fibres during processing). The textile industry has widely and generally accepted the use of enzymes in its processes, especially in fairly simple large-scale applications, such as stone-washing (Sørup *et al.* 1998). The market share of industrial enzymes in the textile field stands at about 10% of the total market, being textile enzymes one of the fastest growing areas in the enzyme industry (BCC 1998; Sørup *et al*. 1998).

Enzymes can be applied potentially to all stages of textile production. The most classical enzymatic application in the textile industry is the desizing of cotton fabrics using amylases (Nilsson and López-Ainaga 1996). The use of amylases to remove starch-based sizing agents has decreased the use of harsh chemicals in the textile industry, resulting in a lower discharge of waste chemicals into the environment, improving the safety of working conditions for textile workers and has raised the quality of the fabric (Nilsson and López-Ainaga 1996; Sørup *et al*. 1998; Cegarra and Emer 1999).

The following step in the textile process comprises the removal of dirt and impurities from the fabrics (scourig). The application of several enzymes such as

pectinases, lipases and cellulases to perform a bio-scouring has been reported. Pectinases have been used together with cellulases in the elimination of impurities in cotton and wool (Heine 2002; Tzanov *et al*. 2001; Lange 2000; Durden *et al*. 2001). Lipases are used in the textile industry to assist in the removal of size lubricants, and are also used in detergent formulations together with proteases and amylases (Chaplin and Bucke 1990; Cortez *et al*. 2005), being responsible for removing fatty substances from clothing. There is also an increasing use of cellulases in domestic washing products (Cavaco-Paulo *et al*. 1999), where they are claimed to aid detergency and to remove damaged fibrillar material, improving fabric appearance, softness and colour brightness (Chaplin and Bucke 1990).

Most of the fabrics are then subjected to a bleaching procedure, using hydrogen peroxide. The use of catalases to break down residual hydrogen peroxide after the bleaching process of cotton is an already established application (Heine 2002; Jensen 2000; Sørup *et al*. 1998), enabling the dyeing process to be carried out without prolonged washing between bleaching and dyeing, thus being more environmental friendly by saving water, time and energy (Costa *et al*. 2001).

The contamination arising from a dyeing process is concerning, since colour is the first contaminant to be recognized in wastewaters and dyes are difficult to eliminate. Several recent studies report the use of laccases, peroxidases or microorganisms to remove the dyes from the environment and for the decolourization of textiles effluents (Zille *et al*. 2003; Campos 2000; Ramalho *et al*. 2002).

The last stage in a textile process is the finishing, where several enzymes can be applied, depending on the intended look and final properties. A very well established application is the use of cellulases in the replacement of pumice stones in the “stone-washing” process to produce the aged appearance of denim

garments (Nilsson and López-Ainaga 1996; Sariisik 2004; Cavaco-Paulo *et al*. 1998; Tzanov *et al*. 2003a). Cellulases are also used in bio-polishing processes to improve the appearance of cellulosic fabrics (particularly cotton and Lyocell) by removing fuzz fibre and pills from the surface, reducing pilling propensity, or delivering softening benefits (Azevedo 2001; Cavaco-Paulo 1998).

Proteolytic enzymes are used in the textile industry for the degumming of silk and for producing sandwashed effects on silk garments (Lange 2000; Heine 2002; Freddi *et al*. 2003; Sørup *et al*. 1998). The use of proteases has also been reported for cotton scouring (Hsieh and Cram 1999) and to prevent backstaining in denim washing (Yoon *et al*. 2000). The use of proteases to reduce prickle and improve softness in wool has been investigated with encouraging results (Heine 2002).

Research on the use of proteases for decreasing the felting tendency of wool has been carried out since the beginning of the 1900's, but the results obtained so far present a high variance and no great achievements comparing to the classical chemical method. Therefore wool bio-finishing was not yet implemented at an industrial scale (Heine 2002). Some of the recent attempts are described below:

- In the earliest enzyme finishing processes, wool was pre-treated by gas chlorination (Chlorzyme process) or by hydrogen peroxide (Perzyme process) prior to treatment with papain and bisulphite (Heine 2002; Mackinson 1979). These processes resulted in a complete removal of the cuticle cells, but because of non-tolerable weight loss of the fibre and treatment irregularity, these processes were never implemented on an industrial scale.
- From the patent literature it is obvious that proteases can be used for wool

treatment but always with corresponding wool pre-treatment or specific modification to enhance the enzyme action (Dybdal *et al*. 1996; McDewitt and Winkler 1999; Leman 1999; Breier 1999).

- Other patented processes describe methods for wool treatment using proteases and other class of enzymes. The enzymes researched so far to assist proteases in the attenuation of wool shrinkage have been transglutaminases (Ogawa *et al*. 1991; McDewitt and Winkler 2000), peroxidases or oxidases (Yoon 1998) and protein disulphide isomerase (King and Brockway 1989).
- Some patents refer methods to improve the shrink-resistance of wool either by treating wool previously with an oxidizing agent (Kondo *et al*. 1985) or alkali-containing alcohol solution (McDevitt and Shi 2000) and then with a protease. Other authors refer processes to achieve shrink-resistance by treating wool with a protease followed by a heat treatment (Ciampi *et al*. 1996).
- Several authors have suggested the use of benign chemical processes such as low-temperature plasma to treat wool (Chi-way *et al*. 2004; Höcker 2002). Plasma treatment is a dry process, which involves treating wool fibre material with electric gas discharges (so-called plasma). At present, there are obstacles (costs, compatibility and capacity) to large-scale commercialization of a plasma treatment process.

Consequently, the application of proteases for achievement of wool shrink resistance is being extensively researched, but the main problem, still not completely solved, lies in obtaining the anti-felting effect that is comparable to that produced by commercially used chlorine-Hercosett process.

1.4.1 LIMITATIONS OF WOOL PROTEASE FINISHING

Since wool is a protein fibre and proteases are fairly unspecific in their hydrolytic action, many problems arise from the wool hydrolysis using proteases, explaining the poor success of all the attempts for wool bio-finishing using this type of enzymes. The main problem with proteases is their controllability. Excessive protease treatment can severely damage wool, especially if used after an oxidative process.

Generally speaking, if the proteases are applied at levels that provide the sufficient shrink resistance in washing, wool fibres are unacceptably damaged and the treatment is not uniform and regular. Major reasons for such treatments to be unsuccessful are:

- Proteases can diffuse inside wool inducing high levels of strength loss;
- Process control is laborious and not feasible for the wool wet processing, when native proteases are applied;
- No real methodologies have been developed to induce a superficial treatment of wool;
- Wool is a very heterogeneous substrate, changing with the race, sex, age and nutrition of sheep (Pailthorpe 1992; Ülkü *et al*. 1998).

1.5 DESIGN OF EXPERIMENTS

In research and development, often half of the resources are spent on solving optimization problems. With the rapidly rising costs of experiment making, it is essential that the optimization is performed with as few experiments as possible. This is one important reason why design of experiments is needed. Experimental design, more commonly called design of experiments (DOE), is an important statistical tool. DOE is a systematic set of experiments that allows one to evaluate the impact, or effect, of one or more factors without concern for extraneous variables or subjective judgements. Thus, it is a structured, organized method for determining the relationship between the factors affecting a process and the output of that process (Box *et al*. 1978; Lynch 2003; Barros Neto *et al*. 1995). Factors are the variables under investigation that are set to a particular value (level) during the experiment. These variables may be quantitative or qualitative. Response variables are the results from the experimental run. An understanding of the relationship between the response variables and the factors is the desired outcome of the entire DOE effort. Once the relationship is understood the response variable can usually be optimized by setting the factors to their optimal levels.

Typical examples were this methodology can be applied are:

- the development of new products and processes;
- optimizing the quality and performance of an existing product and
- optimizing existing manufacturing processes in several industrial areas, like chemicals, polymers, drugs and pharmaceuticals, foods and beverages, cosmetics, and so forth.

The objective of statistical methods is to make a process as efficient as possible (Box *et al*. 1978).Thus, the optimization of a process or a product involves setting the factors so that the output becomes "as good as possible". Often this is done by changing one separate factor at a time until no further improvement is achieved. The One-Factor-At-a-Time approach (OFAT) is, however, very inefficient. As shown by the English statistician Ronald Fisher (Fisher 1925), changing one factor at a time does not give any information about the position of the optimum where there are interactions between factors, which occurs frequently (Fisher 1925; Anderson 2005). Then the OFAT approach gets blocked, usually far from the real optimum. However, the experimenter perceives that the optimum has been reached because changing one factor at a time does not lead to any further improvement.

In 1925, Ronald Fisher introduced the full factorial experiment to study the effect of multiple variables simultaneously, thus starting the development of methods of design of experiments. In his early applications, Fisher wanted to find out how much rain, water, fertilizer, sunshine, etc. were needed to produce the best crop. Since then, much development of the technique has taken place and these methods have been further refined by Box and Hunter (Box *et al*. 1978), Taguchi (Taguchi 1987), and others, so that today they comprise a tool box for virtually any optimization problem, and are in use in just about every industry (Anonymous 2005).

The DOE process consists of four primary phases: the planning phase, the screening phase, the optimization phase and the confirmation phase (Lynch 2003; Anderson 2005). The planning phase is often minimized by most experimenters

despite being a vital component to achieve the desired results from the DOE effort. The purpose of this phase is to clearly define the target and objectives of the experiment, obtain an understanding of all the potential variables and devise a conscious strategy on how to address them in experimentation. In DOE there are three potential objectives: maximize, minimize, hit a target and minimize variation. No variables should be omitted at this point, even if minimal impact on the analysis is anticipated. Once identified, it is important to classify all of the variables as controllable or uncontrollable and to define a conscious choice strategy for addressing each variable.

The second phase of the DOE process is the screening phase. The goal of the screening phase is simply to identify the variables (factors) that have a significant effect on the response. A secondary goal is efficiency. The screening process should be accomplished as cost effectively and quickly as possible. During the screening phase, the focus is not on the development of a mathematical model, but on understanding the few potentially significant variables that have an effect on the response. Screening design results often leads to further experimentation as the cause and effect relationship is progressively revealed.

The third phase of the DOE process is the optimization phase. The purpose of the optimization phase is to take the input from the screening phase and determine optimal factor level settings that generate the desired response. This can be accomplished by the development of a mathematical model or by means of using iterative approaches, such as simplex methods. Both approaches have advantages and disadvantages.

The final phase in the DOE process is the confirmation phase. The purpose

of the confirmation phase is to ensure that the results from the DOE analysis correlate with the actual process. This phase is critical to verify the effectiveness of the predictive power of the results and to ensure their reliability. This confirmation is typically performed by operating the process at the optimal factor level settings, suggested by the analysis and comparing the actual process response results with the predicted response from the analysis.

Thus, there are a number of DOE strategies that vary in complexity and nature. These strategies include full factorial and fractional factorial designs, response surface methodologies, simplex designs, among others. Carefully planned, statistically designed experiments offer clear advantages over traditional OFAT alternatives. These techniques are particularly useful tools for process validation, where the effects of various factors on the process must be determined. Not only is the DOE concept easily understood, but also the factorial experiment designs are easy to construct, efficient, and capable of determining interaction effects. Results are easy to interpret and lead to statistically justified conclusions. The designs can be configured to block out extraneous factors or expanded to cover response surface plotting. Those implementing a DOE strategy will find that computer software is an essential tool for developing and running factorial experiments.

1.5.1 Applications of the DOE Strategy to Textile Processes

The DOE strategy has been widely used in evaluating the effects of several variables and in the optimization of several technological processes, such as immobilization and production of enzymes and production of food components,

among others; though this methodology is seldom implemented in textile processes.

Among the few published works on the use of DOE strategies in textile processes, some of them considered wool as the studied material. These works and the achievements they accomplished will be presented briefly bellow.

In a recent work performed by Tzanov *et al*. (2003b), the effects of the process variables (reaction time, enzyme and modifiers concentration) on the wool enzymatic dyeing were evaluated, using a 2^3 factorial design. According to the authors, the adopted statistical techniques demonstrated their usefulness in finding the optimal conditions for the process which renders laccase dyeing an economically attractive alternative to the conventional high water, dyes, auxiliaries and energy consuming acid dyeing of wool.

Also, Jovančić *et al*. (1993) studied the influence of Basolan DC on the changes in the mechanical and physico-chemical characteristics of wool fabric by means of a central composite design. According to their study, the minimum area shrinkage after washing and the least degradation of wool fibres were obtained with a Basolan DC concentration between 2.5 and 4% on the weight of wool.

In another study conducted by the previous author (Jovančić *et al*. 1998), the effect of several variables on the shrink resistance properties of wool, treated with the serine proteinase Bactosol SI, were investigated by means of a central rotatable design. The independent variables included the concentration of the enzyme, the pH of the treatment bath and the treatment time. Enzyme concentration and pH of the treatment bath had a significant effect on wool shrinkage and degree of whiteness. Treatment time also had a marked effect, particularly on the mechanical properties of the woollen knit fabric. The

optimization attained by the authors consisted in using a time of 90 minutes, an enzyme concentration of 4.9-7.2 g/L and pH values between 8.75 and 9.4 in the wool treatment process.

1.6 PERSPECTIVE AND AIMS OF WORK

The classical antifelting treatments for wool assume a chlorine treatment with polymer deposition, which has many ecological drawbacks, as well as some handling and durability disadvantages. Although many attempts have been made to replace this process by an environmental friendly one, the unacceptable weight loss caused by proteolytic attack eliminates the potentiality of these enzymatic finishing methods.

The aim of the present work is to develop an enzyme-based antifelting treatment for wool. The specific objectives intend to increase the molecular size of proteases in order to reduce their diffusitivity on wool, thus controlling the proteolytic attack. The methods used for that purpose include increasing molecular size of proteases with normal crosslinking agents of proteins agents like glutaraldehyde and covalent attachment to soluble-insoluble polymers of high molecular mass.

This strategy represents a new approach for the study of the proteolytic finishing of wool, allowing to overcome some of the most important drawbacks that the previous attempts were facing, such as the penetration of protease inside the wool fibre and the controllability of the enzyme.

CHAPTER 2

TREATMENT OF WOOL FIBRES WITH SUBTILISIN AND SUBTILISIN-PEG

"The first principle is that you must not fool yourself…

and you are the easiest person to fool. "

Richard Feynman (1918–1988),

Nobel Prize in Physics, 1965

2. TREATMENT OF WOOL FIBRES WITH SUBTILISIN AND SUBTILISIN-PEG

2.1 INTRODUCTION

The present chapter analyses and compares the behaviour of two proteases, native subtilisin and polyethylene glycol (PEG)-subtilisin (which differ essentially in their size), in the hydrolytic attack to wool fibres. As a control, to differentiate between the adsorption and diffusion of the enzymes, two water soluble proteins without catalytic activity, namely bovine serum albumin and carbonic anydrase, were used.

The effect of the pre-treatment on enhancing the enzyme adsorption into wool, prior to the enzymatic treatment, was also evaluated.

To monitor the penetration of enzymes inside the wool, several techniques were used; among them, fluorescence microscopy was performed to visualize enzyme distribution in the wool.

The major objective of this chapter was to understand the nature of enzyme-wool interactions which lead to wool degradation, and investigate the possibility of using a modified protease to develop an enzymatic process for wool finishing, which would be an environmental friendly alternative to the conventional chlorine treatments.

2.2 MATERIAL AND METHODS

2.2.1 ENZYMES, PROTEINS AND REAGENTS

The enzymes used in this study were the proteases Subtilisin Carlsberg (Protease type VIII), (E.C.3.4.21.62) and PEG-Subtilisin, a subtilisin that was modified by covalent coupling to polyethylene glycol (6 mol PEG/ mol protein), all acquired from Sigma-Aldrich. The proteins bovine serum albumin (BSA), carbonic anydrase and the chemicals fluorescein isothiocyanate (FITC) were from Sigma. All other reagents used were of analytical grade.

2.2.2 ENZYMATIC ACTIVITY ASSAY

The activity of proteases was measured at 37ºC by following the increase in absorbance at 660 nm with 0.65% casein solution in 50 mM phosphate buffer, pH 7.5 as substrate. After incubation of 1 ml of diluted soluble enzyme (native or modified) for exactly 10 minutes at 37ºC with 5 ml of casein solution, the reaction was stopped by addition of 5 ml of 110 mM TCA solution in water, and the precipitate was removed by filtration and centrifugation. Then, 2 ml of filtrate were mixed with 5 ml of 500 mM Na_2CO_3 solution and 1 ml of two-fold diluted Folin´s reagent. After vigorous mixing, the colour was allowed to develop for 30 min at 37ºC. The amino acids produced were analysed at 660 nm, taking DL-tyrosine as standard. One unit of activity is defined as the amount of enzyme that hydrolyses casein to produce equivalent colour to 1 µmol of tyrosine, per minute, at pH 7.5 and 37ºC (colour by the Folin&Ciocalteu's reagent).

2.2.3 PROTEIN CONCENTRATION

The total protein concentration was determined by a modification of the micro Lowry method (Lowry *et al*. 1951), using bovine serum albumin as standard and using Sigma test kit nº P 5656.

The possible interference of PEG in the estimation of the protein was analysed. For this purpose, standard solutions were prepared (BSA and BSA with 1% PEG) with concentrations in the range of 0.1–0.5 mg/ml, and the absorbance was determined. No significant changes in the absorbance values occurred when PEG was present (data not shown).

2.2.4 FITC LINKAGE TO PROTEINS

Enzymes were linked to FITC (100/1 w/w) in sodium carbonate buffer pH 8.5. The mixture was dialyzed until no release of FITC was verified by spectroscopy. Wool samples were treated in this solution at 37ºC, 100 rpm, for 24 hr. Wool fibres cross-sections were analyzed by a Transmission optic microscope (Olympus BH2) with magnification of 40 x.

2.2.5 TENSILE STRENGTH

Tensile Strength Resistance was determined by using a tensile tester machine, accordingly to ASTM D5035-90. The samples were conditioned before testing in a standard atmosphere. The tensile strength resistance values are given as the mean of 10 replicates, together with the standard deviation (the coefficient of variation was bellow 10% for all cases).

2.2.6 FELTING AND PILLING

Felting and pilling were visually evaluated after repeated washing (3 times) at 50ºC, for 60 min and 20 rpm, using a liquor ratio of 1/20.

2.2.7 SIZE-EXCLUSION CHROMATOGRAPHY (SEC)

The proteins size was determined by size-exclusion chromatography using a UV-detector at 280 nm and a Pharmacia Hi-Prep Sephacryl S-300 HR column (Amersham Pharmacia Biotech). The conditions of the assay were: room temperature; eluent: 50 mM phosphate, 100 mM KCl, pH 6.5 buffer; flow: 2.5 ml/min and sample volume of 1 ml. Tiroglobulin (669 kDa), Apoferritin (443 kDa), β-Amylase (200 kDa), Alcohol dehydrogenase (150 kDa), Bovine albumin (66 kDa) and Carbonic Anydrase (29 kDa) were used for calibration.

2.2.8 ADSORPTION ON WOOL FIBRES

The adsorption experiments were performed in flasks each containing equal amounts of the sorbent material: 0.5 g of 100% woven merino wool fabric or 23 μm (mean diameter) wool yarns, subjected to the different pre-treatments described bellow. Volumes of phosphate buffer solution (pH 7.6, 0.01 M) and protein stock solution were added to the sorbent so that every flask contained the same total volume (50 mL) but different protein concentration. Then, the flasks were closed and rotated end-over-end for 7 days for the wool fabrics and for 24 hours for the wool yarns, at 37ºC and 90 rpm, in a shaking water bath. Several controls were

run simultaneously: a control test with wool without protein (C1), a control test with the highest concentration of protein without wool (C2) and a control test with the highest concentration of protein and 1 mM of antipain, a serine proteases inhibitor (C3). After incubation, wool fabrics or yarns were removed and washed. The remaining solution was centrifuged and the protein concentration in the supernatant and the amount of aminoacids produced in Tyrosine equivalents was measured. The adsorbed amount of protein was calculated from the difference in protein concentration before and after adsorption. All measurements were performed using at least duplicate samples.

2.2.9 PRE-TREATMENTS PERFORMED ON WOOL FIBRES

The pre-treatment washings performed on wool were the following:

- Surfactant Washing (S): wool was washed with Lutensol ON 30 (non-ionic surfactant) 1 g/L, in a bath ratio 1:20, at pH 9.0 (Na_2CO_3 0.1 M e $NaHCO_3$ 0.1 M buffer), for 30 min, at 40ºC, on Rota-wash machine. After the washing procedure, the surfactant was removed from wool first with tap water, followed by distilled water.
- Bleaching Washing (S+B): After the previous washing, wool was immersed in a bath (same bath ratio) with 1% (o.w.f.) H_2O_2, at pH 9.0 (Na_2CO_3 0.1 M e $NaHCO_3$ 0.1 M buffer), for 1 hour at 55ºC, on Rota-Wash machine.

2.3 RESULTS AND DISCUSSION

Proteases can catalyze the degradation of different components of a wool fibre, making reaction control difficult. Figure 2.1 shows damaged wool fibres caused by treatment with Subtilisin, the protease used in this study. It is also possible to see that the proteolytic attack is not uniform, due to the heterogeneity of the wool itself (Rippon 1992).

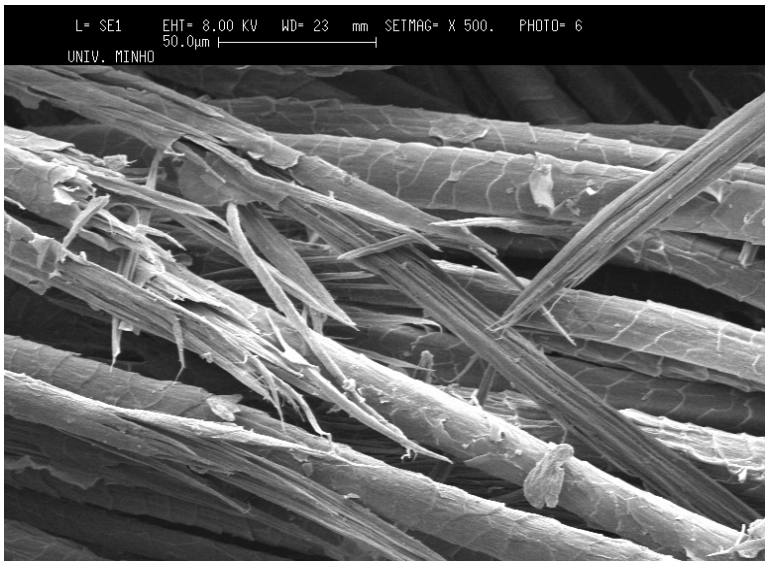


Figure 2.1 – SEM microphotography showing damaged and undamaged wool fibres after treatment with Subtilisin, in pH 7.6 buffer, at 37ºC, for 3 days.

2.3.1 EFFECT OF PRE-TREATMENT

The wool fibres surface is covered by a covalently bound fatty layer, being responsible for the strong hydrophobicity of wool which can be partially removed by alkaline pre-treatments. To test the effect of the pre-treatment on the adsorption of proteins into wool, merino wool fibres with mean diameter of 23 µm were employed. These yarns were subjected to two alkaline pre-treatments in order to enhance the protein penetration inside the fibre. Figure 2.2 shows that there are

no significant changes on wool surface after the pre-treatments performed (surfactant and bleaching washing).

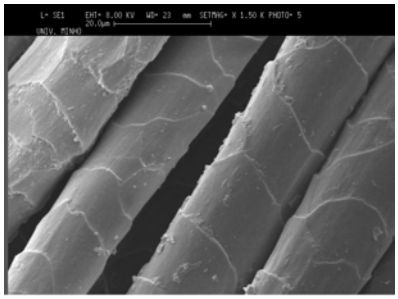
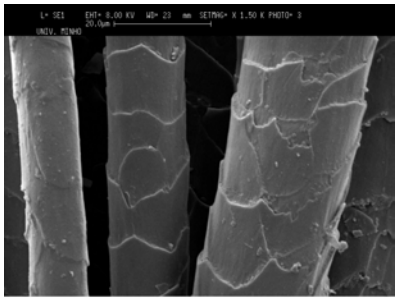
a)  b) 

Figure 2.2 – SEM microphotographs of the wool fibres after the alkaline pre-treatments: a) surfactant washing, and b) surfactant and bleaching washing.

The protease was added at several concentrations to the pre-treated wool yarns, and the experimental adsorption data was fitted by non linear regression analysis to Langmuir model in order to draw the binding curves (Figure 2.3).

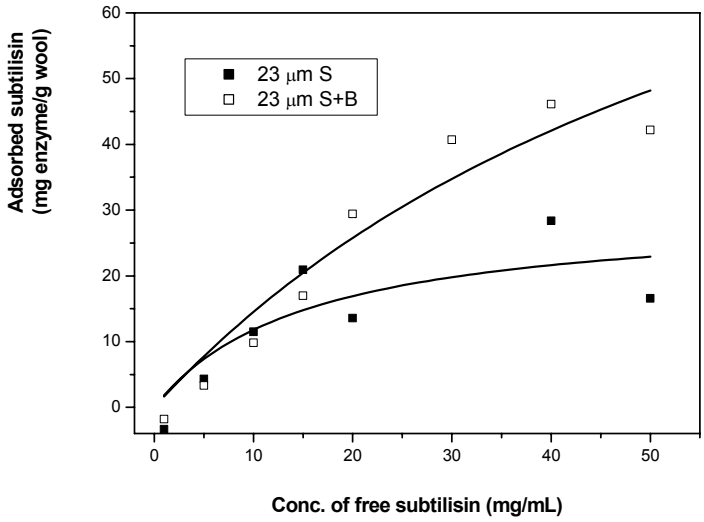


Figure 2.3 – Langmuir isotherm (—, solid line) and experimental data for the adsorption of free subtilisin on 23 μm wool yarns, subjected to a surfactant (S) or a bleaching washing (S+B).

The Langmuir model did not satisfactorily explain the behaviour of protein

adsorption. This was manifested in the poor agreement between the experimental data and simulated curves and also in the low values for the correlation coefficients (data not shown). This was already expected since the wool surface is very heterogeneous, and thus Langmuir model is inadequate to describe adsorption of proteins on this adsorbent.

Observing Figure 2.3 it is possible to see that the amount of adsorbed subtilisin was clearly higher for wool that was subjected to a surfactant washing and posterior bleaching. This fact indicates that this pre-treatment enables a higher penetration of proteases into wool and consequently a higher degradation level. The bleaching step with H_2O_2 is likely to promote a partial removal of the bounded fatty acid barrier of the epicuticle, probably more efficiently than the alkaline treatment with surfactant only. Schäfer (1994), when studying the diffusion of dyestuffs into keratin fibres found that dyestuffs may diffuse quicker into the cortex of bleached wool than into untreated wool because of the cleavage of cystine and the higher fibre swelling. Moreover, Pascual and Julia (2001) reported that the sorption of chitosan into wool was facilitated by an alkaline peroxide treatment. Thus one can conclude that a simple alkaline surfactant washing (scouring) is not enough to remove the fatty bounded layer, decreasing considerably the adsorption capacity on wool fibres, when comparing with the other treatment.

2.3.2 Effect of Enzyme size

The subsequent studies were performed with 100% wool fabric subjected to an alkaline surfactant washing followed by bleaching. The enzymes used were the

native subtilisin and subtilisin-PEG, a commercial preparation acquired from Sigma. The protein concentrations used were low, so that the surface was never saturated with the enzyme. This study was performed using an enzyme concentration of 40 mg/L and for this reason a longer time had to be employed in order to better understand the differences in the behaviour of the two enzymes. Therefore, a study conducted for 168 hours was performed, where protein adsorption and tyrosine formation were monitored (Figure 2.4).

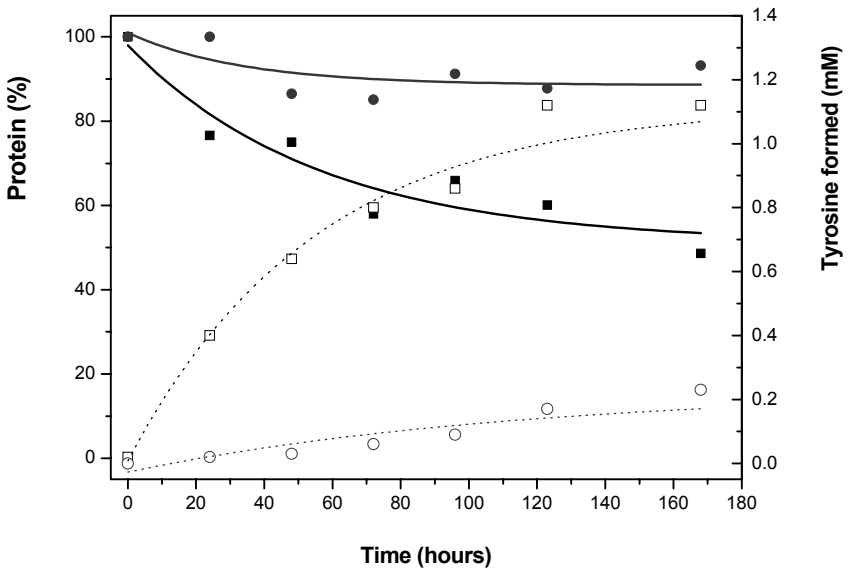


Figure 2.4 – Formation of Tyrosine (mM) (open symbols) and total protein (%) (closed symbols) for the enzymes Subtilisin (■,□) and Subtilisin-PEG (●,○).

The results (Figure 2.4) show that Subtilisin-PEG is not being adsorbed (only about 7% of protein adsorption was attained) while free Subtilisin had about 50% of adsorption into wool fabric. The differences are also noticeable in the formation of Tyrosine equivalents. The subtilisin that was covalently coupled to PEG showed a very low release of aminoacids into media. Comparing to free subtilisin, the amount of aminoacids produced in tyrosine equivalents was much

higher, indicating wool fibre degradation by the enzyme. The control test run simultaneously with free subtilisin and the inhibitor antipain showed no adsorption and no tyrosine formation, confirming that the adsorption of the protease into wool was assisted by the enzymatic action.

This result was also confirmed by the determination of the fibres strength resistance using a dynamometer. The maximum tensile strength supported by the yarns was lower for free subtilisin, indicating higher fibre degradation (Figure 2.5).

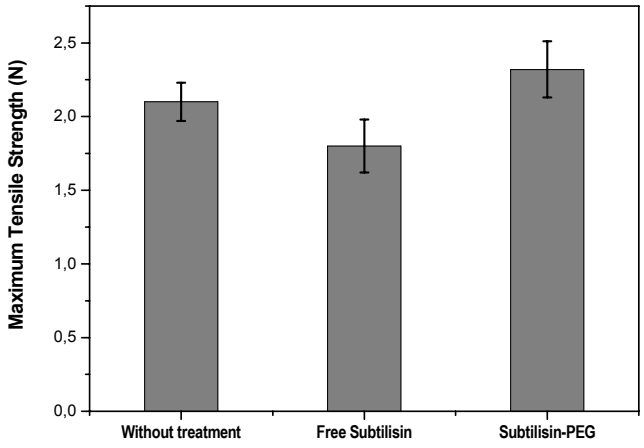
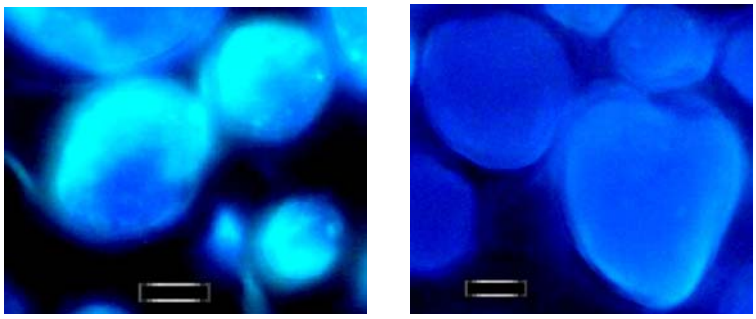


Figure 2.5 - Maximum tensile strength (N) supported by wool yarns without treatment and yarns treated with the same enzyme units of free and modified subtilisin (CV was less than 10%).

To follow the diffusion of the enzymes into fabrics, they were fluorescently labelled with FITC. After covalently coupling the enzymes to a fluorescent dye (FITC), an extensive dialysis was performed until no release of free dye into solution was verified. Then, after enzymatic treatment, a microtome was used to cut thin layers of the fibre entrapped in a non-fluorescent resin. The figure below (Figure 2.6) shows that free subtilisin penetrates completely inside the fibre cortex while fluorescently labelled subtilisin-PEG only appears at the surface of some fibres (in the cuticle layer). A similar result was found by Nolte *et al*. (1996) when

studying the effect of Alcalase, a commercial protease, in wool tops in untreated and Hercosett-treated wool (wool that was treated by the application of a water-soluble resin after chlorination). They found that after a 50 hours treatment, the fluorescently labelled alcalase had fully penetrated the untreated-fibre cortex, while it was retained only at or near the surface of Hercosett-treated fibres after an identical treatment process (Nolte *et al.* 1996). They explained this fact by the temporary barrier to the proteolytic attack provided by the polymer treatment.



a) **b)**

Figure 2.6 – Fluorescence microphotographs of fibre cross-sections of wool treated with FITC-labelled Subtilisin (a) and Subtilsin-PEG (b).

To compare with the adsorption of the different size enzymes, the proteins BSA and Carbonic Anydrase, with average molecular weights of 66 kDa and 29 kDa, respectively, were also tested for adsorption on wool at several concentrations. These two proteins showed no adsorption on wool, thus the isotherms could not be formulated. The following table presents the Langmuir parameters for all tested proteins. As already mentioned, the Langmuir model does not satisfactorily explain the behaviour of protein adsorption, but its parameters were used on a comparative basis, to check for differences in the adsorption and diffusion behaviour of the two enzymes.

Table 2.1 – Values for the relative molecular mass (M*r*) and Langmuir parameters (K_d and Q_{max}), for the several enzymes and proteins tested for adsorption into wool fabrics

Samples	M*r** (kDa)	K_d (g/L)	Q_{max} (mg/g)
Subtilisin	~ 20	93 ± 68	172 ± 89
Sub-PEG	~ 110	---	< minimum conc.
BSA	~ 66	---	< minimum conc.
Carbonic Anydrase	~ 29	---	< minimum conc.

*__M*__*r* are mean values, determined by SEC (Material and Methods section)

In the above table is possible to see that the proteins BSA and Carbonic Anydrase were not adsorbed on wool, no matter their size. As for the enzymes, subtilisin-PEG, the large enzyme, was also not adsorbed. Since wool treatments were performed using the same enzyme units in the bath treatment, it seems that the bigger size of Subtilisin-PEG is responsible for the limitation verified in the proteolytic attack. This could be explained by the restricted accessibility of this enzyme to wool. The large enzyme molecule is not able to enter in contact with substrate and to form the intermediate enzyme-substrate complex, because of steric constraints. It is known that proteases hydrolyze mainly the inside of the fibre rather than cuticle (Sawada and Ueda 2001). This fact is due to the high hydrophobicity of the external surface of wool on one hand and the fatty layer overlapping the cuticles, on the other. Thus, proteases degrade preferentially the intercellular cement, penetrating under favourable conditions relatively quickly into the fibre cortex (Nolte *et al*. 1996). In our study it seems that subtilisin-PEG hydrolyzed just the cuticle layer of wool fibre, explaining the low release of aminoacids and the higher tensile strength resistance of the fibre. To support this idea, wool fibre

samples treated with these two enzymes were washed for 3 consecutive cycles in a rota-wash machine and felting was evaluated visually. It seems that wool fibre treated with subtilisin-PEG felted less (Figure 2.7), highlighting the idea that it had its cuticle layer partially removed.

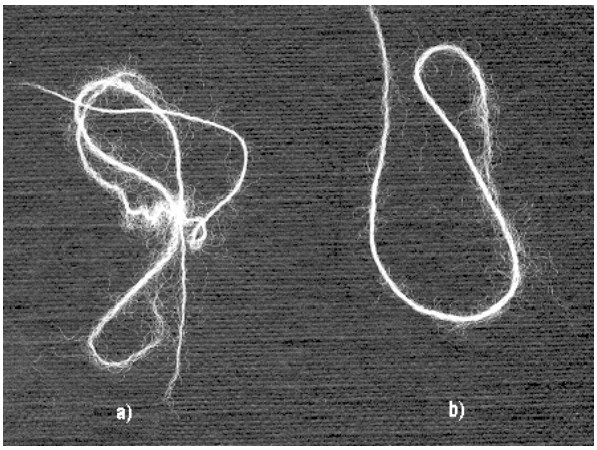


Figure 2.7 - Visual damages on wool yarns after treatment in a Rota-wash machine. Samples: *a)* wool yarn treated with free Subtilisin and *b)* wool yarn treated with Subtilisin-PEG.

This fact could be very useful in wool finishing, where only the cuticle layer is intended to be hydrolyzed. The dimension of the protease is a self-limiting factor for the undesirable hydrolysis of wool fibre cortex, thus overcoming the major drawback of wool enzymatic finishing: the difficulty in controlling enzyme hydrolysis process.

2.4 CONCLUSIONS

The adsorption of a native and a modified subtilisin on wool was studied. The alkaline peroxide pre-treatment improves the enzyme diffusion on wool. This diffusion seems to be facilitated by the hydrolytic attack, since proteins without

activity could not adsorb considerably on wool.

Subtilisin-PEG, the big protease, hydrolyzed just the cuticle layer of wool, fact that was confirmed by the lower release of aminoacids into media and the higher tensile strength and lower felting of the fibre. Thus, the production of diffusion-controlled enzymes might be a solution for a future enzymatic wool treatment process, which would be an environmental friendly alternative to the conventional chlorine treatments.

CHAPTER 3

CHEMICAL MODIFICATIONS ON PROTEINS USING GLUTARALDEHYDE

"That's one small step for man;

One giant leap for mankind."

Neil Armstrong (1930-)

Apollo 11 astronaut

3. CHEMICAL MODIFICATIONS ON PROTEINS USING GLUTARALDEHYDE

3.1 INTRODUCTION

In the previous chapter it was demonstrated that the production of diffusion-controlled enzymes might be a promising solution for an enzymatic wool treatment process. Thus, the intent of the study described in this chapter was to create diffusion-controlled enzymes by crosslinking with the bifunctional compound glutaraldehyde (GTA).

This chapter describes the effect of crosslinking the enzyme Esperase (E.C. 3.4.21.62), a modified Subtilisin used for finishing in textile industry, and the proteins Bovine Serum Albumin and Casein with GTA on molecular weight increase. It was intended to investigate the behaviour of the mentioned proteins modified by glutaraldehyde, such as the formation of dimers and higher oligomers and the production of enzymatic aggregates with preserved activity.

Two common techniques of measuring molecular weight of proteins were used: SEC and SDS-PAGE. Furthermore, the degree of covalent modification of the amino groups was evaluated.

Glutaraldehyde, a bifunctional compound mainly used in chemical modifications of proteins and polymers, links covalently to the amine groups of lysine or hydroxylysine in the protein molecules (Cao *et al.* 2000). The chemical modification of proteins with crosslinking agents can be used for the reinforcement of the compact tertiary structures resulting in protein stabilisation against pH inactivation (Cao *et al.* 2000) and several approaches of chemical modification have also been used to increase the thermostability of proteases, like trypsin, α-

chymotrypsin and subtilisin (He *et al*. 2000).

3.2 MATERIAL AND METHODS

3.2.1 ENZYME, PROTEINS AND REAGENTS

The enzyme used in this study was the protease Esperase, a modified subtilisin (E.C.3.4.21.62) (from Novozymes). The proteins bovine serum albumin and casein (from Sigma) were used as controls. All other reagents used were of analytical grade.

3.2.2 PROCEDURE FOR THE PREPARATION OF BOVINE SERUM ALBUMIN AGGREGATES

A solution of 20 mg mL-1 of Albumin was prepared (A0) in 10 mM sodium acetate and 5 mM calcium acetate buffer pH 7.5. Aggregation of the molecules was induced by slow addition of glutaraldehyde (from Aldrich, 50% fresh solution in water) to the clear solution under gentle stirring at 4ºC for 2 hours. Several solutions were prepared containing 0.06% (v/v), 0.13% (v/v) and 0.25% (v/v) of glutaraldehyde in solution, and the samples were labelled as A0.06, A0.13 and A0.25, respectively.

3.2.3 PROCEDURE FOR THE PREPARATION OF CASEIN AGGREGATES

A solution of 5 mg mL-1 of casein was prepared (C0) in 50 mM potassium

phosphate buffer pH 7.5. Aggregation of the molecules was induced by slow addition of glutaraldehyde (from Aldrich, 50% fresh solution in water) to the clear solution under gentle stirring at 4ºC for 2 hours. Several solutions were prepared containing 0.01% (v/v), 0.02% (v/v), 0.04% (v/v), 0.08% (v/v) and 0.25% (v/v) of glutaraldehyde in solution, and the samples were labelled as C0.01, C0.04, C0.08 and C0.25, respectively.

3.2.4 PROCEDURE FOR THE PREPARATION OF ENZYME AGGREGATES

The enzyme aggregates were prepared using a solution of 20 mg mL-1 of the enzyme Esperase and 50 μM antipain, a protease reversible inhibitor, in 10 mM sodium acetate and 5 mM calcium acetate buffer pH 7.5 (E0). To the solution, 1 mM CaCl2 was also added. A fresh solution of 1% glutaraldehyde was added slowly under gentle stirring at 4ºC, until reaching the final concentrations of 0.01% (v/v), 0.02% (v/v), 0.04% (v/v), 0.06% (v/v) and 0.20% (v/v), being the samples labelled as E0.01, E0.02, E0.04, E0.06 and E0.20.

3.2.5 GEL ELECTROPHORESIS

To separate the proteins and to determine their molecular weights, SDS-PAGE was carried out using the Hoefer miniVe system from Amersham Pharmacia Biotech. The resolving gels (10% acrylamide of about 1.5 mm thickness) were run at a constant voltage (120 V) and prepared according to the method originally described by Laemmli (Laemmli 1970). The current was stopped when the bromophenol blue dye marker had reached about 1 cm from the bottom of the gel. Following electrophoresis, to observe the protein-banding pattern on the

gel, staining was carried out either by using Coomassie blue or silver staining. Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic Anydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α– lactalbumin (14.4 kDa) were used for calibration.

3.2.6 SIZE-EXCLUSION CHROMATOGRAPHY

The protein size was determined by size-exclusion chromatography, as previously described in section 2.2.7.

3.2.7 DEGREE OF COVALENT MODIFICATION

The method used for determining the modification of proteins at their amino groups is the modified assay of Morçöl (Morçöl *et al*. 1997) in which the primary amines on proteins react with the sodium salt of trinitrobenzenesulfonic acid (TNBS). Unmodified (or native) proteins were used as standards in the experiments. Modified proteins preparations and the protein standards were diluted in the concentration range of 1 mg/ml to 0.05 mg/ml in 0.1 M borate buffer containing 0.15 M NaCl, pH 8.0. Two ml of the samples were mixed with 50 μl of 30 mM aqueous TNBS solution and the mixture was incubated for 30 min at room temperature. The blank for the assay consisted of 2 ml of buffer. The absorbances of modified and unmodified proteins were read against the blank at 420 nm and the data were plotted as a function of increasing protein concentration. The degree of covalent (irreversible) modification at amino groups was calculated using the formula: % covalent modification = [(A-B) / A] x 100, where A and B are the slopes

of the unmodified standard and modified protein, respectively, as determined from the absorbance data at 420 nm in the linear regime.

3.2.8 Enzyme assay and Protein Concentration

The activity of proteases was measured accordingly to the procedure described in section 2.2.2.

The total protein concentration was determined by the Bradford (Bradford 1976) method, using bovine serum albumin as standard.

3.3 Results and Discussion

To accomplish this study, two proteins were chosen as model proteins, by the fact that they are widespread and low cost, and being so, the ideal compounds for study. These proteins were a globular protein, Bovine Serum Albumin (BSA), having a molecular weight close to 66 kDa and a flexible protein, Casein (CAS), whose molecular weight is near 23 kDa. This milk protein was also chosen because it has a molecular weight close to the enzyme that was intended to study, the Esperase.

3.3.1 Preparation of Casein aggregates

Casein aggregates formation was verified by the analysis of the chromatogram attained by SEC. The results are shown in Figure 3.1.

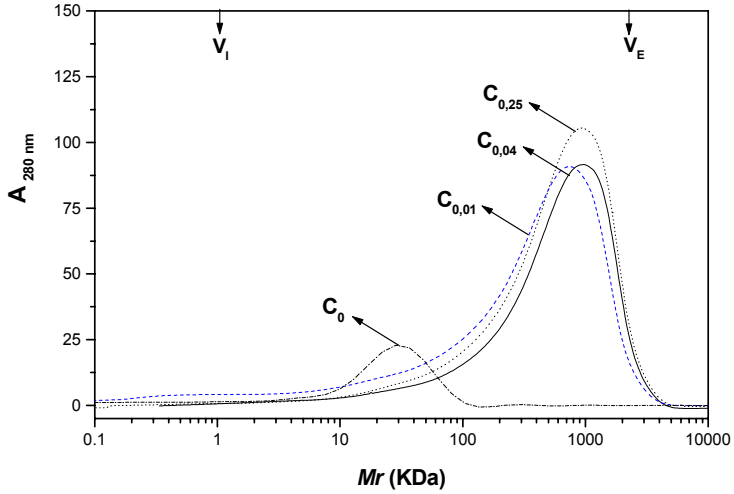


Figure 3.1 - Size-exclusion chromatography elution patterns of the native (C_0) and modified Casein, on 50 mM potassium phosphate buffer pH 7.5. Modified samples were labelled as $C_{0.01}$ – casein solution with 0.01% GTA (v/v); $C_{0.04}$ – casein solution with 0.04% GTA (v/v) and $C_{0.25}$ – casein solution with 0.25% GTA (v/v). The scale was modified from elution time (t_E) to molecular mass (in Da) using the calibration equation: $Log\ Mr = 7.85815 - 1.65159 \times ((t_E \times 2.5)/103.62)$.

As can be seen in Figure 3.1, after adding just 0.01% (v/v) GTA to the solution, an aggregate with high molecular mass is formed (curve $C_{0.01}$ on chromatogram). This aggregate is about 40 fold the native casein size (C_0). Adding higher concentrations of GTA does not change the chromatogram profile significantly, once this aggregate appears in the same chromatogram zone, very close to the column exclusion volume. It was expected that with the addition of low GTA concentrations, the formation of dimers or other oligomers of less Mr occurred. Instead, big agglomerates were formed. Because this was not expected to occur, complementary assays were performed using SDS-PAGE. In this technique, an anionic detergent is used (sodium dodecylsulphate - SDS), to disrupt secondary and tertiary structures of protein molecules, and weak

interactions among them.

As can be seen in Figure 3.2, defined multimers of casein are formed with increasing glutaraldehyde concentration. At the lowest concentration of 0.01% glutaraldehyde (lane B in Figure 3.2) it is possible to observe the formation of higher complexes that are trapped at the interface of the stacking gel and the running gel. The dominant band is shifted to a position corresponding to the trimeric form of casein. However, higher complexes appear as well, migrating slower in the gel. The dominant band starts to be smoother and it is possible to see the higher multimeric complexes trapped at the interface of the gels. On lane E (0.08 % GTA), all the casein complexes got trapped in the stacking gel. This pattern of crosslinking was not detected in size-exclusion chromatography, where all samples were presented as a multimeric complex of about 40 times the weight of casein. This may be due to the action of SDS that disrupts aggregated proteins non-linked by covalent bonds, whereas in HPLC those formations are eluted in a non-disrupted form.

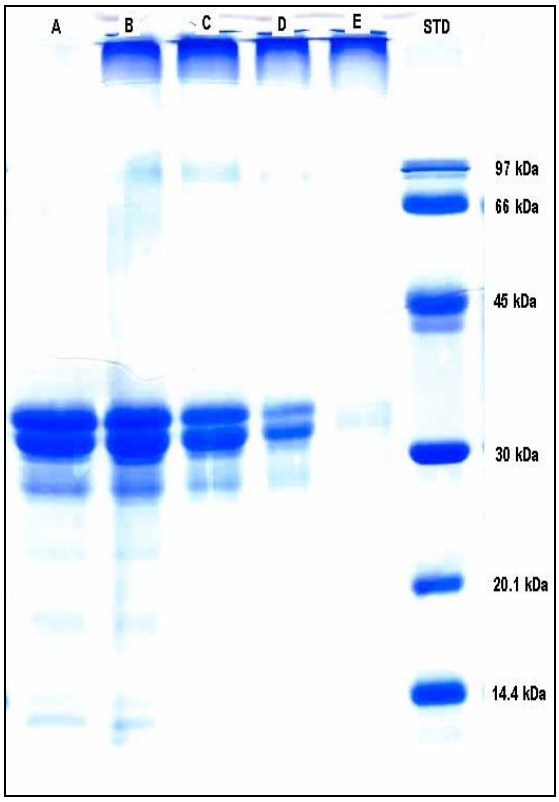


Figure 3.2 – SDS-PAGE of the samples of casein. Lanes: A - no addition of glutaraldehyde (native casein), B - casein with 0.01% (v/v) GTA, C - casein with 0.02% (v/v) GTA, D - casein with 0.04% (v/v) GTA, E- casein with 0.08% (v/v) GTA and STD – molecular mass markers.

3.3.2 PREPARATION OF ALBUMIN AGGREGATES

In Figure 3.3 the chromatogram attained by SEC to BSA is presented. Analysing this figure it may be seen that with the addition of 0.06% (v/v) GTA to the protein solution, dimer formation occurs, this is, two unmodified Albumin molecules aggregate, having thus twice the molecular weight of native (unmodified) BSA. This molecular mass increase is gradual with increasing final GTA concentration in solution, seen in Figure 3.3 by the curve shifts towards higher molecular weights from $A_{0.06}$ to $A_{0.25}$. When 0.25% (v/v) of GTA is added to the solution (curve $A_{0.25}$ on chromatogram), a big protein agglomerate is formed,

having about 20 fold the size of unmodified Albumin, and elutes near the exclusion column volume, determined by Blue Dextran. It is interesting to see that when this same concentration of GTA was added all at once (curve $A_{0.25*}$ on chromatogram), the chromatographic profile was very different, presenting two main inflexions of the curve. It is believed that the first peak corresponds to GTA that has not reacted with the protein. It is well known that this compound can promote self-oligomerization, explaining the molecular mass near 1 kDa.

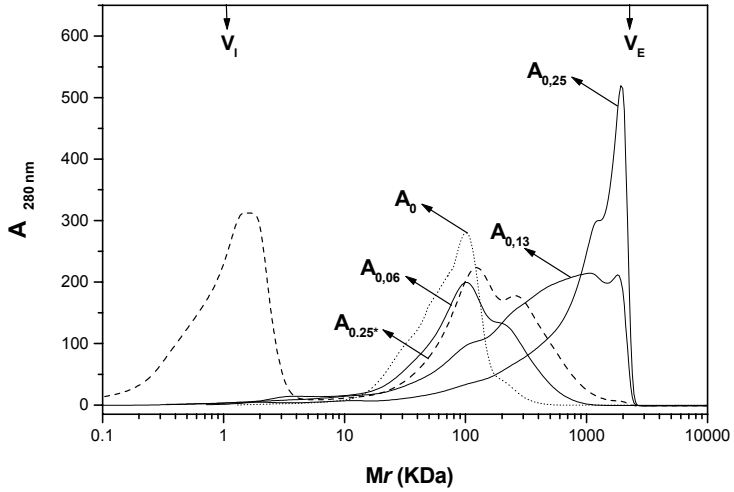


Figure 3.3 – Size-exclusion chromatography elution patterns of the native (A_0) and modified Albumin on sodium and calcium acetate buffer pH 7.5. Modified samples were labelled as $A_{0.06}$ – albumin solution with 0.06% GTA (v/v); $A_{0.13}$ – albumin solution with 0.13% GTA (v/v) and $A_{0.25}$ – albumin solution with 0.25% GTA (v/v). The scale was modified from elution time (t_E) to molecular mass (in Da) using the calibration equation: $Log\ Mr = 7.85815 - 1.65159 \times ((t_E \times 2.5)/103.62)$.

In the second inflexion of the curve it is possible to see the peak of free BSA and its dimers (that is, BSA increased twice). This data leads us to the assumption that GTA reacts promptly with free lysine groups of proteins and, when

in excess, it partially polymerises to give oligomers. For this reason, to attain protein oligomers of high molecular weight it is advisable to add the bifunctional reagent slowly, in small amounts. The mechanism of multimers formation may be a two-step process, first by the reaction of a monomer or oligomer with the crosslinker and second, by the reaction of this oligomer/monomer containing a crosslinker with a monomer lacking a crosslinker (a “free” monomer). This reaction may be a covalent linking type or just an electrostatic one. When GTA is added all at once, it links to all available lysine groups and then, perhaps by some phenomenon of steric hindrance, it polymerises to give GTA oligomers.

3.3.3 Preparation of Enzyme aggregates

Similar experiments were conducted with Esperase, a commercial Subtilisin from Novozymes. In the chromatographic study performed with Esperase, the GTA concentrations were kept below 0.20% (v/v), in order to keep the enzyme active (data not shown).

In Figure 3.4 the chromatographic profile for Esperase modification by GTA is presented. Analysing this Figure, it is seen that no increase in molecular weight occurs, despite the use of antipain, a protease inhibitor, to prevent auto proteolysis.

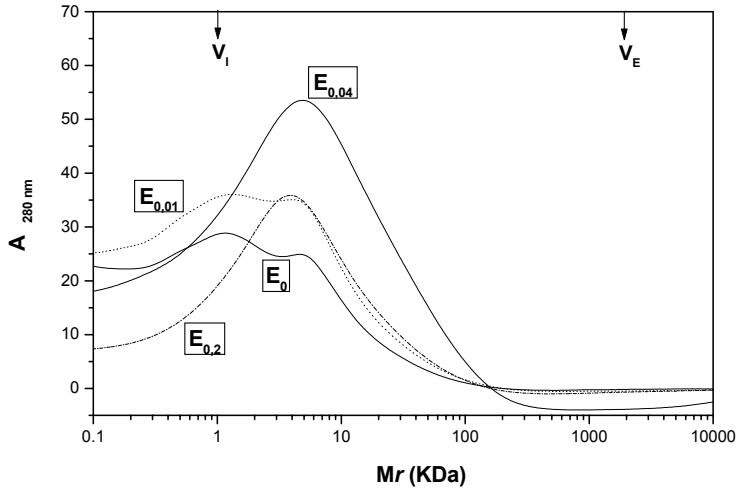


Figure 3.4 - Size-exclusion chromatography elution patterns of the native (E_0) and modified Esperase on sodium and calcium acetate buffer pH 7.5. Modified samples were labelled as $E_{0.01}$ – esperase solution with 0.01% GTA (v/v); $E_{0.04}$ – esperase solution with 0.04% GTA (v/v) and $E_{0.20}$ – esperase solution with 0.20% GTA (v/v). The scale was modified from elution time (t_E) to molecular mass (in Da) using the calibration equation: $Log\ Mr = 7.55643 - 1.43295 \times ((t_E \times 2.0)/98.062)$.

The low concentration of GTA added in order to maintain enzyme active may have had influence on the poor enzymatic aggregation verified. Another plausible explanation is that Esperase, being a commercial preparation, contains other compounds used as stabilisers that may have reacted preferentially with GTA. The amount of total protein present in the enzymatic preparation is in the order of 7%, a value considered normal when compared to other commercial preparations, but indicative of the high amount of stabilisers and/or additives in solution. It is, therefore, important that all amines or other compounds that may react with GTA should be removed prior to aggregation, in order to enhance the efficiency of GTA crosslinking.

3.3.4 EXTENT OF CROSSLINKING

In Figure 3.5, the degrees of covalent modification of amino groups, calculated by the modified TNBS method, are presented for both proteins and Esperase. The correlation coefficients of the plots were 0.98 or higher for both native and modified proteins.

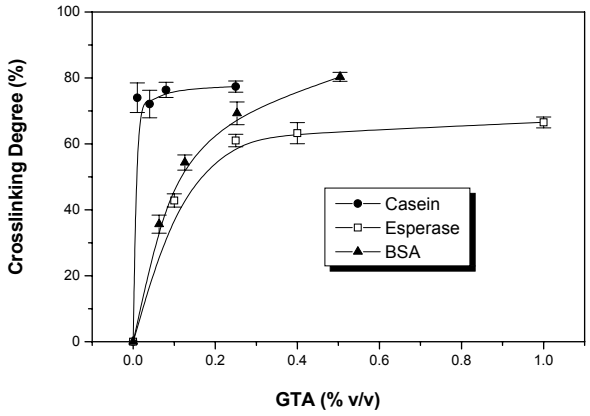


Figure 3.5 – Extent of crosslinking of BSA, Casein and Esperase as a function of glutaraldehyde (GTA) concentration.

In case of Casein, with low amounts of GTA (0.01% v/v), the degree of covalent modification was $74 \pm 4\%$. Adding more GTA had only a slight effect on this degree, confirming the results of SEC and SDS-PAGE. For BSA, this increase in the crosslinking degree was less pronounced, and with 0.25% (v/v) of GTA added, it presented the value of $69 \pm 3\%$. When the concentration of GTA doubled, this value ranged $80 \pm 1\%$, and was still increasing. Once again, this test confirmed the results attained by SEC, showing that aggregates formation with BSA is a slower process that with casein.

Values in the same magnitude were also obtained by Bigi *et al*. (2001) who

found that with 0.25% GTA, the degree of crosslinking on gelatine films was about 85% and increased to near 100% when GTA concentrations above 1% were used.

For Esperase, it may be seen that the degree of covalent modification of amino groups using the bifunctional reagent glutaraldehyde occurs in a much slower way. With 0.25% (v/v) of GTA added to the enzymatic solution, the degree of covalent modification achieved for Esperase was 61 $\pm$ 2%, this value being 16% less than that attained for Casein. The maximum crosslinking degree achieved for Esperase was 66 $\pm$ 2%, when 1.00% (v/v) GTA was used. This crosslinking degree was attained in the enzymatic preparation, which contains about 7% of protein (i.e. enzyme), so it may correspond to the modification of other amino groups present in solution or other substances that may have reacted with GTA.

The number of available amino groups able to interact with GTA in each tested protein was investigated (Table 3.1). The proteins Albumin and Casein have 60 and 26 free amino groups, respectively, able to link covalently to glutaraldehyde (these free amino groups are originated from the ε–amino groups of lysine residues and a terminal α–amino group).

Table 3.1 – Content of Lysine residues in the proteins tested (from Protein Data Bank, PDB)

PROTEIN	LYSINE RESIDUES
Esperase (P29600)	5
Casein (P02663)	25
Bovine Serum Albumin (CAA76847)	59

With regard to the protease Esperase, it has only 5 available lysine residues (see Table 3.1). This fact, by itself, explains the poor aggregation verified with this enzyme. The problem of the non-accessibility of amino groups to attack by

glutaraldehyde should not be a valid explanation since the ionised side-chains, like those of Lys and Asp, tend to be on the exterior of the enzymes and able to interact with the solvent (Price and Stevens 1999).

3.4 CONCLUSIONS

The results presented confirm that the number of free lysine groups is a key issue in the formation of soluble aggregates when the bifunctional reagent used for crosslinking is glutaraldehyde. In proteins with a high amount of free lysine residues, glutaraldehyde crosslinking constitutes an effective way of multimers formation. The different reactivity of glutaraldehyde on BSA and Casein can be associated with the specific conformation of each protein. Casein is a relatively small protein with a flexible open structure, and thus the access of GTA to its lysine residues is facilitated comparing to BSA, a globular protein, which has restricted accessibility of lysine residues for reaction.

It was found that the increase in Mr was gradual with increasing final glutaraldehyde concentration in the solution. Interestingly, the way in which GTA was added was also important. It was verified that to attain protein oligomers of high molecular mass it is advisable to add the bifunctional reagent slowly and in small amounts. The explanation for this may reside in the fact that GTA reacts promptly with the available lysine residues, and then, perhaps by some phenomenon of steric hindrance, it self polymerises to give GTA oligomers.

SDS-PAGE confirmed the aggregate formation attained in chromatography. It also showed that despite the formation of dimers and trimers, it is not always possible to see their existence by chromatography because they elute in a non-

disrupted form, which means, in the same elution time. Note that this chromatographic study was conducted without the addition of SDS to the elution buffer.

The modified TNBS method also confirmed the results of SEC and SDS-PAGE, showing the quick agglomerate formation for casein, even with low amounts of GTA.

For Esperase, TNBS method showed a much slower reaction between GTA and enzyme. Actually, maximum covalent modification of amino groups attained for Esperase was 66%, when a high concentration of GTA was used (1% v/v). It is thought that this value may correspond to the covalent modification of amino groups of other compounds eventually present in the enzymatic solution. It was seen that GTA concentrations above 0.20% promoted high losses of enzymatic activity. For this reason, the chromatographic study performed with the enzyme used concentrations below that value. This study revealed that no agglomerate formation was found for Esperase using this bifunctional compound. A valid explanation for this fact should be the low amount of lysine groups available for crosslinking in Esperase.

These chromatographic results show that glutaraldehyde in not an adequate crosslinker for this enzymatic class. Other bifunctional compounds, able to interact with other enzyme reactive groups should be used, to increase its molecular mass to the desired values.

CHAPTER 4

THE USE OF REVERSIBLY WATER-SOLUBLE IMMOBILIZED PROTEASES FOR WOOL TREATMENT

"The art of discovery consists in seeing what everyone else has seen and then thinking what nobody else has thought"

A. Szent Györgyi (1893–1986),

Nobel Prize for Physiology or Medicine, 1937

4. THE USE OF REVERSIBLY WATER-SOLUBLE IMMOBILIZED PROTEASES FOR WOOL TREATMENT

4.1 INTRODUCTION

The immobilization of proteases on solid supports by covalent attachment can offer several advantages over the free enzyme including easy handling, recovery from the reaction medium and reuse and/or operation in continuous reactors (Ferreira *et al*. 2003). Though, the proper interaction of the enzyme with a solid substrate like wool would only occur if the enzyme is in a soluble state. This heterogeneous enzymatic system would be more effective and interesting from an industrial point of view if one could recover the enzyme after the treatment. Recently, the use of soluble-insoluble matrices for enzyme immobilization is being studied, due to the many advantages of this system (Rodrigues *et al*. 2002; Sardar *et al*. 2000; Arasaratnam *et al*. 2000). One of these such matrices is Eudragit S-100 which is a polymer that can be reversibly made soluble-insoluble by changing the pH, thus making possible the recycling of the enzymes, a major advantage over other methods which use soluble enzymatic matrices.

In this chapter, the covalent immobilization of a commercial protease to the soluble-insoluble polymer Eudragit S-100, by carbodiimide coupling is described. The stability and activity of the enzymatic conjugate under various storage and operational conditions was evaluated and compared with the native enzyme. Wool enzymatic treatment with both free and immobilized enzyme was studied. Some physical tests on wool fabrics were performed afterwards to compare the treatments and evaluate the wool quality. The viability of this enzymatic wool bio-finishing process using the reversibly soluble protease was investigated.

4.2 MATERIAL AND METHODS

4.2.1 ENZYME AND REAGENTS

The enzyme used in this study was the alkaline protease Esperase (kindly supplied by Novozymes) (E.C.3.4.21.62). Eudragit S-100 was a generous gift from Degussa-Hüls, S.A., Barcelona. Carbodiimide hydrochloride (EDC) and ethanolamine were purchased from Sigma (St. Louis, USA). All other chemicals used were of analytical grade.

4.2.2 ENZYME ASSAY AND PROTEIN CONCENTRATION

The activity of Esperase was measured accordingly to the procedure described in section 2.2.2.

The total protein concentration was determined as described previously in section 2.2.3.

4.2.3 EFFECT OF PH AND TEMPERATURE ON ENZYME ACTIVITY

The effect of temperature and pH on the activity of native and immobilized Esperase was tested. The enzymes were incubated at different temperatures (from 20º to 100ºC, in 0.3 M Tris-Cl buffer containing 0.03 M CaCl2, pH 7.6) and pH solutions (from 4 to 12, using Britton-Robinson buffer with μ=0.3 M at 37ºC). The residual activity was then measured according to the method described in section 2.2.2.

4.2.4 STABILITY MEASUREMENTS

The free and the immobilized enzyme were placed in the refrigerator for storage stability at 4ºC and kept at room temperature for storage stability at ca. 20ºC (RT). The remaining activity was measured after several days of incubation.

To measure operational stability, the native and immobilized Esperase were placed in a water bath at 37ºC or 60ºC and 90 rpm of stirring and the remaining activity was measured at 37ºC (normal temperature of the activity method), after several hours of incubation at the working temperature. At 37ºC, the operational stability was determined at two different pH's: 7.6 and 10.0.

The stabilization factor (SF) was calculated as the ratio between the half-life of the immobilized enzyme and that of the corresponding soluble enzyme.

4.2.5 REUSABILITY

The initial activity of the immobilized enzyme was measured and the conjugate was then subjected to 5 cycles of repeated use. After each run the immobilized enzyme was recovered by lowering the pH to 4.5, centrifugation, alternative washing of the precipitated polymer with acetate buffer pH 4.5 and phosphate buffer pH 7.2 and re-dissolution in Tris-Cl buffer, pH 7.6. The activity was measured after the 1st, 3rd and 5th cycles.

4.2.6 IMMOBILIZATION METHOD

The protease was covalently linked to Eudragit S-100 by carbodiimide coupling using a solution of 2.5% (w/v) of Eudragit S-100 in phosphate buffer pH 7.2 with 0.28 M of NaCl. The pH was raised to 11.3 by the addition of a NaOH solution and then reduced to pH 7.2 with an HCl solution. To the polymer, a 0.2% (w/v) carbodiimide solution was added while mixing for 10 min. Then the enzyme was added (1%, in v/v). This solution was kept under stirring for 1 hour at room temperature and then was mixed with 0.05% (v/v) of an ethanolamine solution (0.45 g/mL) for 1 hour at room temperature. The pH of the mixture was reduced to 4.5 with acetic acid. Precipitated eudragit-enzyme was separated by centrifugation (13000 x g, 10 min) and washed alternatively with 0.01 M acetate buffer containing 0.14 M NaCl (pH 4.5) and 0.02 M phosphate buffer containing 0.14 M NaCl (pH 7.2). Washing was carried out by precipitation at pH 4.5, resuspending in the respective buffers mixing for 10 min, and reprecipitation. Finally, the eudragit-enzyme precipitate was redissolved in 100 mL of 0.3 M Tris-Cl buffer containing 0.03 M $CaCl_2$ (pH 7.6).

4.2.7 GEL ELECTROPHORESIS

To determine the molecular weights of the proteins, SDS-PAGE was carried out according to the procedure described previously in section 3.2.5. Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), and Trypsin inhibitor (20.1 kDa) were used for calibration.

4.2.8 WOOL PRE-TREATMENTS AND ENZYMATIC TREATMENTS

Woven 100% wool fabrics (Albano Antunes Morgado Lda, Portugal) were subjected to a surfactant (scouring) or a surfactant and peroxide washing (bleaching) in order to enhance the proteolytic attack. Wool was washed with 1 g/L non-ionic surfactant Lutensol ON 30 (BASF, Germany), in a bath ratio 1:20, at pH 9.0 (Na_2CO_3 0.1 M and $NaHCO_3$ 0.1 M buffer), for 30 min, at 40ºC, in a Rota-wash machine (MKII Series 7227, Shirley Developments Ltd, England). After the washing procedure, the surfactant was removed from fabric first with tap water, followed by distilled water. For the bleaching step, the wool fabrics were afterwards immersed in a bath (same bath ratio) with 1% (o.w.f.) H_2O_2, at pH 9.0 (Na_2CO_3 0.1 M and $NaHCO_3$ 0.1 M buffer), for 1 hour at 55ºC, in a Rota-Wash machine. Finally, the wool fabrics were abundantly washed with distilled water and allowed to air dry.

The enzymatic treatment for the weight loss determination was performed using 7.2 g of wool fabric in 100 mL of 0.3 M Tris-Cl buffer with 0.03 M $CaCl_2$, pH 7.6 (bath ratio 1:14). The native or modified Esperase was added in order to have approximately 100 U of enzyme activity in the final solution. Treatments were conducted at 37ºC, 90 rpm of stirring, for 72 hours. Wool fabrics were then subjected to 3 machine washing cycles, according to standard EN 26330. The final weight loss was measured after the machine washings.

For the tensile strength and shrinkage tests, the enzymatic treatment was performed at 65ºC, 40 rpm of stirring, during 4 hours, using a liquor ratio of 8.5 g wool fabric in 400 mL of 0.02 M sodium tetraborate buffer, pH 8.5. Enzyme was added in increasing amounts of activity to the bath treatment.

4.2.9 WEIGHT LOSS

Wool fabrics were conditioned at 100ºC for 2 hours, desiccated and weighted until constant weight (considered as differences between successive weights inferior to 1 mg). Assays were performed in duplicate.

4.2.10 TENSILE STRENGHT RESISTANCE AND DIMENSIONAL STABILITY

Tensile strength test was carried out using SDL tensile tester equipped with a load cell maximum capacity of 2 kgf. Also, 100 mm/min of test speed, 10 mm of gauge length and 71.4 Tex of linear density was applied. The measurement of area shrinkage of fabric after washing was according to Woolmark method TM 31.

4.3 RESULTS AND DISCUSSION

Enzymatic wool finishing using proteases is a complex process to implement at an industrial level due to several factors, like wool heterogeneity (wool varies with animal, source, feeding, etc...) and the difficulty in controlling the enzyme hydrolysis. Thus, by immobilizing the enzymes in soluble matrices, one could overcome some of these problems, making process control feasible and easy (Silva and Cavaco-Paulo 2003).

Wool fabrics were subjected to treatments with the native and the modified protease Esperase, and some physical tests were performed to evaluate changes or improvements in wool fabric quality. Also, the physical-chemical properties of the native/modified enzyme were studied.

4.3.1 EFFECT OF PH AND TEMPERATURE ON ENZYME ACTIVITY

Figure 4.1 shows the effect of reaction pH and temperature on the relative activity of the immobilized and native Esperase. As shown in Figure 4.1a), maximum enzyme activity was observed in the alkaline region, as expected for proteases from *Bacillus sp*. (Banerjee *et al*. 1999). The optimal pH is shifted from about 9.5 to 10.5 pH units upon immobilization. In earlier studies, an increase in optimum pH was observed with papain immobilized on an enteric polymer (Fujimura *et al*. 1987), chymotrypsin immobilized on Eudragit S-100 (Sharma *et al*. 2003), and an alkaline protease immobilized on vermiculite (Chellapandian 1998). This change in optimum pH is usually explained by an alteration in the microenvironment of the enzyme due to immobilization or support. Sharma (Sharma *et al*. 2003) explained this pH shift toward the alkaline region by the anionic nature of the matrix.

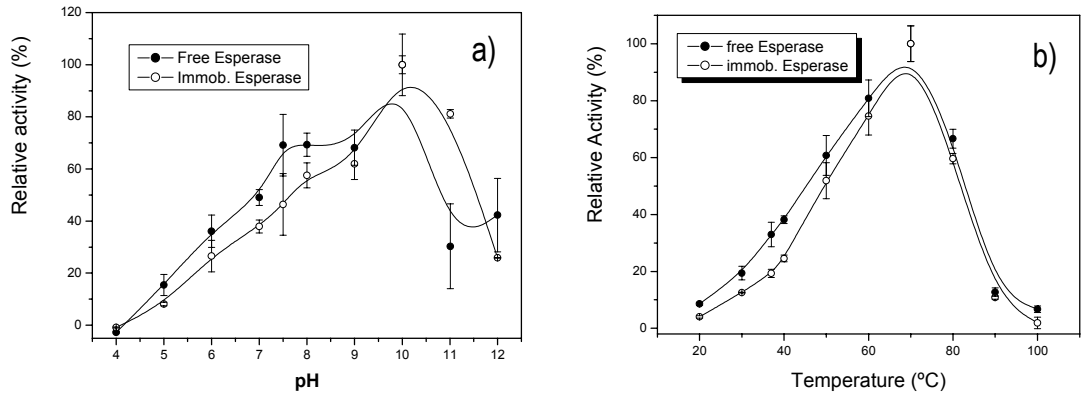


Figure 4.1 – Effect of reaction pH (a) and temperature (b) on the relative activity of free and immobilized Esperase.

The activity of free and immobilized Esperase in Eudragit S-100 increased up to 70ºC and then decreased with further increases in temperature (Figure 4.1b). Normally an increase in temperature increases enzyme activity up to a maximum

level and thereafter a decline in activity is observed due to the denaturation of the protein. So, the optimum temperature for Esperase was found to be around 70ºC for both enzymatic forms. Therefore, the chemical coupling of the enzyme to the smart polymer seems not to change its temperature profile. Similar results were obtained with an alkaline protease immobilized on vermiculite (Chellapandian 1998).

4.3.2 OPERATIONAL AND STORAGE STABILITIES OF THE ENZYME

The thermal stability of enzymes is one of the most important features for the application of the biocatalyst from a commercial point of view. This parameter was evaluated at 4ºC, room temperature, 37ºC and 60ºC. Table 4.1 summarize the results attained.

Table 4.1 – Half-life times ($t_{1/2}$) for the native and modified Esperase at several temperatures

Enzyme	Temperature	Free	Immobilized	SF[a)]
Esperase	4ºC	140 ± 33 days	770 ± 260 days	5.5
	RT	8 ± 1 days	54 ± 10 days	6.8
	37ºC (pH 7.6)	1.4 ± 0.2 days	19 ± 2 days	13.6
	37ºC (pH 10)	5.0 ± 0.6 days	17 ± 2 days	3.4
	60ºC	0.58 ± 0.04 hours	7.3 ± 0.5 hours	12.6

a) Stabilization factor (SF) as a ratio of half-life times.

It is interesting to note that there was a significant decrease in inactivation of the immobilized enzyme when compared to the free enzyme. The stabilization factor was considerably high, ranging from 3.4 to 13.6.

At 4ºC (storage stability on refrigerator) the enzyme is quite stable ($t_{1/2}$ for native Esperase is 140 days). Upon immobilization, the enzyme saw its half-life

time increased about 5 fold. At room temperature, the proteases stability decreases considerably, as expected. Again, the immobilization brought high increases in $t_{1/2}$ (stabilization factor is 6.8).

At 37ºC, the half-life time for the native and immobilized Esperase was evaluated at pH 7.6 and 10.0. It was observed that Esperase was significantly more stable at pH 10.0 than at pH 7.6. This result confirms the alkaline nature of this enzyme. The immobilized Esperase presented a considerably higher stability at pH 7.6 (the stabilization factor is 13.6, confirming that the pH shift verified to the alkaline side is a result of the nature of the matrix). At pH 10.0, the increase in the stabilization factor was smaller, as already expected, given that this is the enzyme optimum pH and thus no considerable changes will arise at this pH.

The operational stability at 60ºC was greatly improved upon immobilization. After 0.6 hours at this temperature native Esperase had only half of its initial activity, while modified Esperase still retained around 98% of its initial activity ($t_{1/2}$ at 60ºC is 7.3 hours).

Thus, in this work, it may be seen that the thermal inactivation of immobilized Esperase is much lower than that of native Esperase, both at low and high temperatures. The improved stability of immobilized enzymes over their soluble counterparts may be related to the prevention of autolysis (it is known that immobilization of proteases is able to reduce autolysis (Sharma *et al*. 2003) and thermal denaturation (He *et al*. 2000; Ferreira *et al*. 2003). The immobilization of the enzyme causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation by raising the temperature. In conclusion, the immobilized proteases present an improved thermal stability over its soluble counterparts and may be attractive biocatalysts for industrial purposes.

4.3.3 KINETICS OF ENZYME REACTIONS

The catalytic activity of the soluble and immobilized Esperase was accessed using casein as the substrate. In all the cases, Michaelis-Menten kinetics was observed and its parameters were determined, in order to evaluate the substrate specificity of the immobilized preparations. To that purpose, the enzymes (free and immobilized) were incubated with increasing concentrations of the substrate. This high molecular weight substrate was used to a better understanding and representation of the interactions among the proteases and wool (a big substrate).

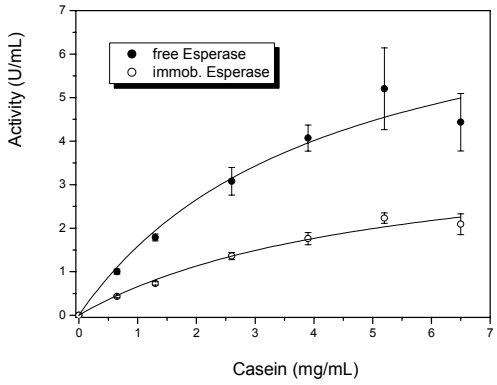


Figure 4.2 – Changes in enzyme activity at different substrate (casein) concentrations.

The Michaelis-Menten parameters for the native and immobilized enzyme, at 37ºC, were attained by the hyperbolic regression and are listed in Table 4.2. The V_{max} values decreased upon immobilization (from 8.2 to 4.0 U/mL) while K_M values increased upon immobilization (from 4.2 to 5.2 mg/mL). Increases in K_M

values upon immobilization have been frequently reported when the matrix is insoluble (Zhou and Chen 2001) or soluble (Zacchigna et al. 1998), but also with these smart polymers. An increase in K_M values was observed when trypsin was coupled to eudragit S-100 (Arasaratnam *et al*. 2000), and when xylanase was immobilized on the same polymer, increases in K_M values from 3.6 mg/ml to 5,0 mg/ml for the substrate xylan were verified (Sardar *et al*. 2000).

Comparison of the K_M value for a given free and immobilized enzyme provides information about the interaction between enzyme and its support. An increase in K_M once an enzyme has been immobilized indicates that the immobilized enzyme has an apparent lower affinity for its substrate than that of the free enzyme, which may be caused by the steric hindrance of the active site by the support, or the loss of enzyme flexibility necessary for substrate binding. So, the V_{max} value of immobilized enzyme is lower than that of the free enzyme.

Table 4.2 – Kinetic parameters for casein hydrolysis with free and immobilized Esperase (determined by hyperbolic regression)

Enzyme	Esperase	
	V_{max} (U/mL)	**K_M (mg/mL)**
Free	8.2 ± 1.7	4.2 ± 1.7
Immobilized	4.0 ± 0.7	5.2 ± 1.6

4.3.4 MOLECULAR WEIGHT DETERMINATION

To evaluate the molecular weight of the enzymatic preparations, an SDS-PAGE electrophoresis was carried out. The gel attained by this procedure is shown below (Figure 4.3). The immobilized Esperase presents a big diffuse band of high molecular weight, which stays at the beginning of the resolving gel. This

band presents a molecular weight above 97 kDa (the highest standard). Also, on this sample, the band of the free enzyme is still present, showing that some enzyme may be merely adsorbed to eudragit. Some studies report that this non covalently bounded enzyme can be removed with additional washing steps using surfactants like Triton X-100 (Rodrigues *et al*. 2002; Arasaratnam *et al*. 2000; Silva *et al*. 2005). This test confirms the higher molecular weight of the immobilized Esperase over the soluble form.

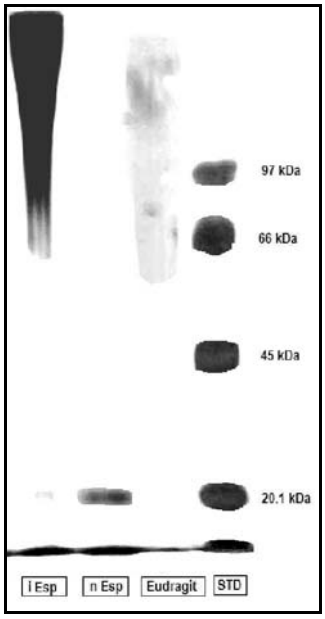


Figure 4.3 – SDS/PAGE of the native and modified Esperase. Lane 1, modified Esperase, Lane 2, native Esperase, Lane 3, Eudragit alone and Lane 4, molecular-mass markers.

4.3.5 REUSABILITY OF ENZYMATIC PREPARATIONS

The reusability of the alkaline protease immobilized on Eudragit S-100 has also been studied because of its importance for repeated use in industrial processes. The decrease in activity on repeated use of immobilized Esperase is

given in Figure 4.4. The Eudragit-Esperase conjugate retained 72% of its original activity after five cycles of repeated uses, showing a high stability.

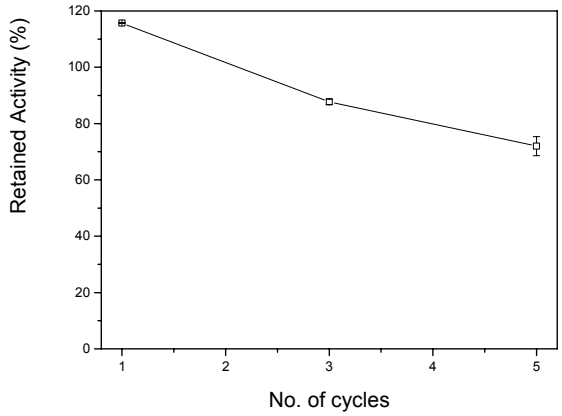


Figure 4.4 – Retained activity of the immobilized Esperase (in %) after several cycles of repeated use (initial activity was taken as 100%).

4.3.6 WOOL TREATMENTS

Prior to the enzymatic treatments, the wool fabric was subjected to a washing procedure using surfactant (scouring) or surfactant and hydrogen peroxide (bleaching), in order to improve the contact of the enzyme with its substrate (wool). The fabrics were then treated with the native and immobilized Esperase. Two enzymatic treatments were performed: one in harsh conditions, for weight loss determination, at 37ºC, pH 7.6 for 72 hours followed by 3 machine washing cycles and one in smoother conditions for the tensile strength and shrinkage tests at 65ºC, pH 8.5 during 4 hours.

In the following figure, the weight losses caused by the enzymatic treatment on wool fabrics are shown. For each enzyme (native or immobilized), the combined effect of the enzymatic treatment together with the pre-treatment (bleaching) was evaluated after the 3 consecutive machine washing cycles.

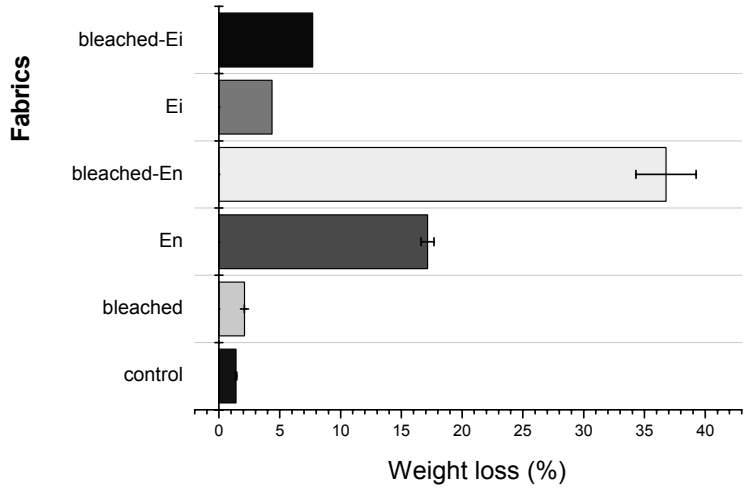


Figure 4.5 – Percentage final weight loss of the wool fabrics subjected to the pre-treatment and the enzymatic treatment, followed by 3 machine washing cycles. Control is wool without any wet treatment, followed by 3 machine washing cycles (En - native Esperase and Ei - immobilized Esperase).

The fabrics were treated using the same enzyme units in the water bath. This means that the effect of the enzymes is directly related and compared. Wool fabric treated with free Esperase and subjected to 3 washing cycles presents the worst weight loss (about 37% when enzymatic treatment was applied after bleaching and about 17% when no pre-treatment was used). The immobilized enzyme did considerably less damage to the wool fabric (the weight losses are in the order of 4% and 7%, respectively, for the same conditions). Also, the damage caused by the pre-treatment alone is insignificant (weight loss less than 2%) and can be neglected.

This test also confirms that the pre-treatment makes the substrate (wool) more accessible to the proteolytic attack, since the weight losses verified were always higher for the wool fabrics that were pre-treated before the enzymatic

treatment. In the case of native Esperase, this difference is more than double. The fabric was extremely degraded after this treatment in severe conditions, but it was our intent to verify if a harsh treatment would completely degrade the fabric. Interestingly, using the exact same treatment conditions and the same activity units of immobilized Esperase, the final weight loss was only in the order of 7%, which is a really significant difference. This confirms the viability in using this immobilized protease for the purpose of wool finishing, using adequate treatment conditions in a way that the desired effects are achieved.

The wool fabrics were also subjected to tensile strength and shrinkage tests, after a moderate enzymatic treatment. Figure 4.6 shows the results of these parameters on wool fabrics treated with increasing amounts of enzyme activity.

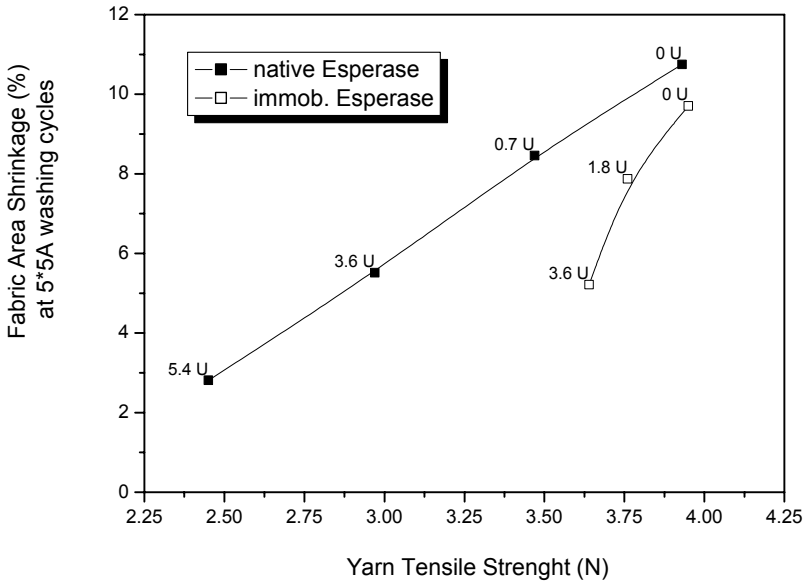


Figure 4.6 – Effect of enzyme treatment with native or modified Esperase on scoured wool fabrics with increasing amounts of enzyme (measured as total enzyme units in the bath treatment).

Analysing the figure above it is possible to see that when 3.6 U of activity were used in the wool enzymatic treatment, an area shrinkage of about 5% was achieved, both for free and immobilized Esperase. This means a reduction to about half of the initial shrinkage. The main differences, however, were verified in the fabric resistance, since with the immobilized Esperase the resistance was still 92% of the original one, while the native enzyme promoted a loss of about 25% in the original tensile strength of the wool fabric. The more native enzyme added to the bath treatment, the more intensive is the tensile strength loss of the fabric. This fact is not verified in the fabric treated with the immobilized form (an almost vertical line is observed). This means that when treating wool with the immobilized Esperase, by the proper choice of treatment conditions, one can achieve good levels of shrink resistance without considerably damaging the fabric. The weight loss was in the order of 3% for the wool treated with immobilized Esperase (data not shown).

It seems that, even when the immobilized enzymes were used to treat wool, some of the fibres presented degradation, explaining the fabrics shrinkage and weight loss. This confirmation was attained by SEM microscopy of wool fabrics after the severe treatment, which shows that some of the fibres treated with immobilized Esperase are degraded (see Figure 4.7). Nevertheless, this degradation was significantly inferior to that verified with the free enzymes, meaning that the impact of degradation can be controlled by the conditions of treatment. The explanation for this degradation may lie in the fact that there is still free enzyme in the immobilized matrices. This noncovalently bounded enzyme can be removed by additional washing steps prior to the wool enzymatic treatment, as

already referred. The fibers that were not degraded presented their cuticle layer removed, thus they have their properties improved.

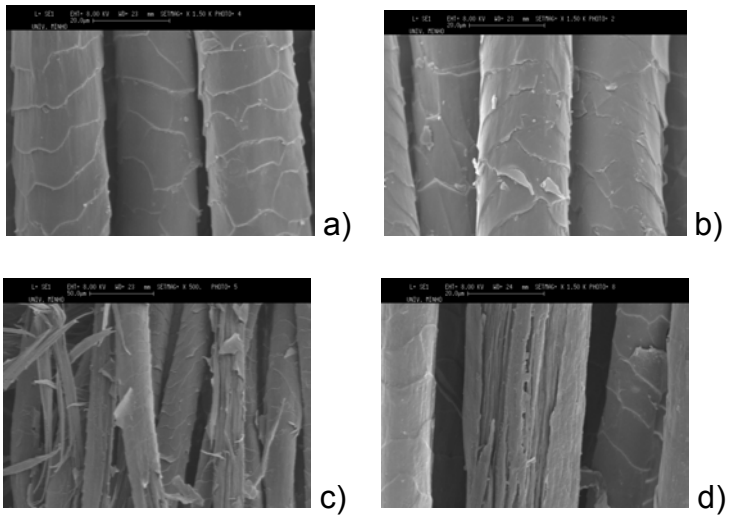


Figure 4.7 - SEM microphotographs of wool fabrics after the treatments: a) Control; b) Bleaching; c) Free Esperase; d) Immobilised Esperase. All the enzymatic treatments were performed using the same enzyme units in the bath (about 100 U).

4.4 CONCLUSIONS

A commercial available alkaline protease was coupled to eudragit S-100, a polymer that can be made soluble-insoluble, by covalent binding using carbodiimide. The optimum pH of the immobilized enzyme was shifted to a higher value, but the optimum temperature was unchanged. The operational and storage stability of Esperase was considerably improved by immobilization. The decrease in the Michaelis-Menten parameters indicate the existence of steric hindrance effects, but since the conjugate is considerably more stable and shows a high reusability, the immobilized preparations are interesting from an industrial point of view. The wool fabrics treated with immobilized Esperase showed a lower weight

loss and a considerably higher tensile strength resistance than the fabrics treated with the native enzyme.

Besides the simplicity of this immobilization method, these enzyme conjugates are a promising approach for wool bio-finishing processes, since they can remove wool cuticles, improving wool properties and can overcome the wool finishing problems with soluble proteases and the environmental problems with the wool chemical treatments. Furthermore, the polymer used has several attractive features which favour its use as an immobilization matrix for enzymes. It is non-toxic (enteric polymer), water soluble, recoverable from solution by altering pH, economical and commercially available (Kumar and Gupta 1998). This process needs to be further characterized to a complete understanding and optimization.

CHAPTER 5

OPTIMIZATION OF A SERINE PROTEASE COUPLING TO EUDRAGIT S-100 BY EXPERIMENTAL DESIGN TECHNIQUES

"My goal is simple. It is a complete understanding of the Universe, why it is as it is and why it exists at all. "

Stephen Hawking (1942–),

Physicist and Mathematician

5. OPTIMIZATION OF A SERINE PROTEASE COUPLING TO EUDRAGIT S-100 BY EXPERIMENTAL DESIGN TECHNIQUES

5.1 INTRODUCTION

In the preliminary tests performed by the research group, the coupling of a commercial protease (Protex Multiplus L) to Eudragit S-100 by the carbodiimide method for the purpose of wool finishing, provided a preparation with a low activity yield and low stability. Thus, the immobilization protocol needed to be optimized for this specific enzyme, creating a more attractive conjugate for industrial application. Some studies report the coupling optimization of several enzymes to Eudragit S-100, using however, the one-factor-at-a-time approach (Rodrigues *et al*. 2002; Tyagi *et al*. 1998; Arasaratnam et al. 2000).

Two-level factorial designs are ideal for identifying the few vital variables that significantly affect the process, and have been applied successfully to study and optimize a different number of biocatalytic and bioseparation processes (Silva and Roberto 1999; Serralha *et al*. 2004; Moyo *et al*. 2003; Cortez *et al*. 2004; Mayerhoff *et al*. 2004).

In this chapter a full factorial design was adopted to study the influence of four different variables, namely polymer concentration, carbodiimide concentration, time of reaction and blocking agent concentration, on the coupling of a serine protease into a soluble-insoluble polymer (Eudragit S-100). The major advantage of studying the influence of several parameters by means of factorial design methodology is to distinguish possible interactions among factors, which would not be possible by classical experimental methods, like the one-factor-at-a-time approach (Box *et al*. 1978).

5.2 MATERIAL AND METHODS

5.2.1 ENZYME AND REAGENTS

The enzyme used in this study was the alkaline serine protease Protex Multiplus L, a modified subtilisin (E.C.3.4.21.62) kindly supplied by Genencor. Eudragit S-100 (MW 135000 composed by 1:2 copolymer of methacrylic acid and methyl methacrylate) is a commercial product from Rhöm Pharma (Darmstadt, Germany). Carbodiimide hydrochloride (EDC) and ethanolamine were purchased from Sigma (St. Louis, USA). All other chemicals used were of analytical grade.

5.2.2 IMMOBILIZATION METHOD

The protease was covalently linked to Eudragit S-100 by the carbodiimide coupling by following a protocol based on Arasaratnam (Arasaratnam *et al.* 2000). A solution (% in w/v) of Eudragit S-100 in phosphate buffer pH 7.2 with 0.28 M of NaCl was used. Its pH was raised to 11 by the addition of a NaOH solution and then reduced to pH 7.2 with an HCl solution. To the polymer, a carbodiimide solution (% in w/v) was added while mixing for 10 min. Then a volume of 1% (in v/v) of the enzyme was added. This solution was kept under stirring for 1-5 hours at room temperature and then was mixed with a blocking solution (ethanolamine 0.45 g/mL) (% in v/v) for 1 hour at room temperature. The pH of the mixture was reduced to 4.5 with acetic acid. Precipitated eudragit-enzyme was separated by centrifugation (13000 x *g,* 10 min) and washed alternatively with 0.01 M acetate buffer containing 0.14 M NaCl (pH 4.5), 0.02 M phosphate buffer containing

0.14 M NaCl (pH 7.2) and washed twice with 0.15 M Tris-3 g/L Triton X-100 buffer containing 0.015 M $CaCl_2$ (pH 7.6). Washing was carried out by precipitation at pH 4.5, re-suspending in the respective buffers mixing for 10 min, and re-precipitation. Finally, the eudragit-enzyme precipitate was redissolved in 100 mL of 0.3 M Tris buffer containing 0.03 M $CaCl_2$ (pH 7.6).

5.2.3 EXPERIMENTAL DESIGN

Four variables, which were expected to have effect on the protease coupling to Eudragit, were identified by a preliminary search of literature. The range and the levels of the variables investigated in this study are given in Table 5.1 and were chosen to encompass the range in literature. The variables considered for the design were: eudragit concentration (A), carbodiimide concentration (B), contact time (C) and ethanolamine concentration (D) and their influence was evaluated according to a 2^4 full factorial design with 4 repetitions at the central point (Table 5.2). For statistical calculations, the variables were coded according to Eq. (5.1):

$$xi = \frac{Xi - X0}{\Delta Xi} \qquad (5.1)$$

where xi is the independent variable coded value, Xi the independent variable real value, $X0$ the independent variable real value on the center point and ΔXi is the step change value. The runs were conducted randomly.

Table 5.1 – Factor levels used according to the 2^4 factorial design

Variables	Levels		
	-1	0	+1
A: Eudragit (% w/v)	0.5	1.5	2.5
B: Carbodiimide (% w/v)	0.2	0.6	1.0
C: Time (hr)	1	3	5
D: Blocking agent (%	0.050	0.325	0.600

The "Design expert" version 5.0 (Stat-Ease Inc., Minneapolis, USA) was used for regression and graphical analyses of the data obtained. The conjugate activity (CA), the operational stability at 60ºC (OS) and the remaining activity after 5 cycles of repeated use of the enzymes (R_5) where taken as the responses of the design experiments. The statistical significance of the regression coefficients was determined by Student's *t*-test and that of the model equation was determined by *Fischer's* test. The proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. The optimum conditions were obtained by the graphical analysis using "design-expert" program.

5.2.4 ENZYME ASSAY AND PROTEIN CONCENTRATION

The activity of proteases was measured according to the procedure described previously in section 2.2.2. The total protein concentration was determined by the procedure described in section 2.2.3.

5.2.5 GEL ELECTROPHORESIS

SDS-PAGE was carried out according to the procedure described in section 3.2.5.

5.2.6 OPERATIONAL STABILITY AT 60°C

The native and immobilized enzymes were placed in a water bath at 60°C and 100 rpm of stirring and the remaining activity was measured after 5 hours of incubation at this temperature.

5.2.7 REUSABILITY

The initial activity of the immobilized enzymes was measured. They were then subjected to 5 cycles of precipitation/dissolution and the activity was measured again. The cycles consisted in precipitating the polymer by lowering the pH to 4.5, centrifugation, alternative washing of the precipitated polymer with acetate buffer pH 4.5 and phosphate buffer pH 7.2 and re-dissolution in Tris-Cl buffer, pH 7.6. The activity was measured after the 1^{st}, 3^{rd} and 5^{th} cycles.

5.3 RESULTS AND DISCUSSION

A commercial protease was coupled to Eudragit S-100 after incubation of the polymer solution with a carbodiimide (EDC) for carboxyl group activation. The enzyme concentration was kept constant during the study and the amount of polymer added was varied. The carbodiimide and blocking agent concentration

and the coupling time were also varied. The activity of the final polymer conjugate containing the immobilized protease was measured at pH 7.6. At this pH, Eudragit S-100 is in a soluble form. Table 5.2 shows the designed experimental matrix and its results.

Table 5.2 - Values for conjugate activity (CA), operational stability at 60ºC (OS) and reusability (R_5), according to the 2^4 factorial design

Assay	Variables				Responses		
	A	B	C	D	CA (U/mL)	OS (%)	R_5 (%)
1	-1	-1	-1	-1	3.69	51.2	13.7
2	+1	-1	-1	-1	4.70	49.8	72.0
3	-1	+1	-1	-1	1.08	58.3	36.8
4	+1	+1	-1	-1	2.60	55.0	67.5
5	-1	-1	+1	-1	3.28	56.0	22.8
6	+1	-1	+1	-1	4.33	51.7	76.0
7	-1	+1	+1	-1	0.77	37.2	44.3
8	+1	+1	+1	-1	1.61	82.5	63.4
9	-1	-1	-1	+1	2.92	60.6	16.5
10	+1	-1	-1	+1	4.22	58.6	78.0
11	-1	+1	-1	+1	0.46	75.3	60.2
12	+1	+1	-1	+1	1.25	137.5	75.7
13	-1	-1	+1	+1	2.67	50.0	19.3
14	+1	-1	+1	+1	3.93	56.2	75.5
15	-1	+1	+1	+1	0.46	83.3	55.6
16	+1	+1	+1	+1	1.19	105.6	85.0
17	0	0	0	0	1.92	74.3	55.0
18	0	0	0	0	1.97	77.4	49.6
19	0	0	0	0	2.04	59.8	44.2
20	0	0	0	0	2.17	62.5	44.0

After the immobilization procedure according to the variations of the factors imposed by the design, the immobilized samples of Protex Multiplus L were

analysed using a gel electrophoresis, to verify the formation of macromolecular aggregates and if there were major differences in their molecular weights. Figure 5.1 shows the results of the SDS-PAGE performed. In this technique, an anionic detergent is used (sodium dodecylsulphate-SDS) to disrupt secondary and tertiary structures of protein molecules and weak interactions among them, thus retaining only the primary amino acid structure of the protein. It is possible to see that in some of the assays the bands of native enzyme are clearly marked (A1, A3, A8, A13-A16) while in others these bands are faded (A4-A7, A9-A11) or not present (A2, A12), indicating less native enzyme or no native enzyme at all in the conjugate. These differences in the protein-banding pattern cannot be attributed to the concentration of protein in the immobilized enzymes since high protein yields, measured as the ratio between conjugate and initial protein amount (data not shown), were attained. Assay 2 had, for instance, a protein yield of 73% and lacks the band of native enzyme. Since the SDS-PAGE was performed on the immobilized samples after being subjected to an extensive washing step using high salt concentrations and surfactant, the merely adsorbed protein was washed out and thus it may be concluded that the enzyme was in this case covalently crosslinked to the polymer. Several authors have reported that different salts and surfactants remove protein non-covalently bound to Eudragit polymers (Tyagi *et al*. 1998; Arasaratnam *et al*. 2000). In assays 1 and 3 there is still native enzyme in the conjugate that was desorbed by the SDS-PAGE procedure, given that the intermolecular forces that bind the protein to the polymer are very weak (Dourado *et al*. 2002).

In all the immobilized samples it is possible to observe the presence of high complexes that are trapped at the interface of the stacking gel and the running gel.

These high complexes might correspond to the enzyme covalently crosslinked to the polymer that could not enter the 10% acrilamide gel, being trapped at the interface. Figure 5.1 also shows that all immobilized samples had different banding pattern suggesting that all the factors considered were significant to the study.

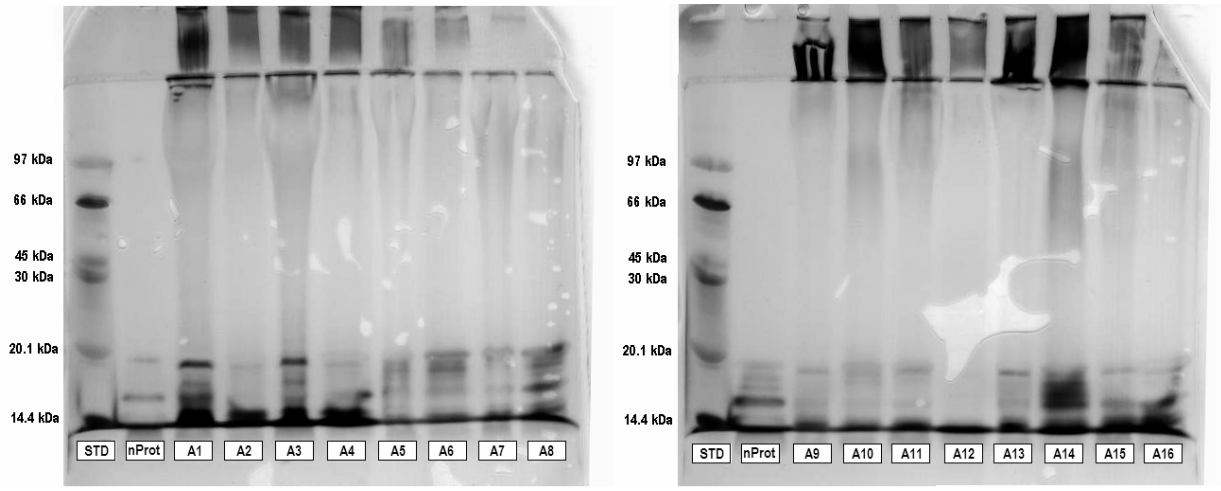


Figure 5.1 – SDS-PAGE electrophoresis of the immobilized enzymes according to the statistical design. Lanes: STD - molecular mass markers, nProt - native Protex Multiplus L, A1 to A16 - assays nº1 to nº16, in the statistical standard order (see Table 5.2).

The statistical analyses for each of the response variables evaluated, namely conjugate activity (CA), operational stability at 60°C (OS), and reusability (R_5), are summarized in Table 5.3. All the four factors studied seem to have played a critical role in the protease immobilization. Table 5.2 shows that the maximum values attained for conjugate activity (above 3,9 U/ml) are found on assays 2, 6, 10 and 14. These assays also have high values for the operational stability at 60°C (above 50%) and reusability (above 72%). These four assays have in common the upper level for eudragit concentration and the lower level for carbodiimide concentration, indicating a tendency in these factors for the maximization of these three responses. The Students *t*-test in Table 5.3 confirms

the higher significance of these two factors on the responses CA and R_5, comparing with the other two factors studied.

According to the Student's t-test results, the concentration of Eudragit, Carbodiimide and Blocking agent presented a significant effect (more than 95% confidence level) for all responses tested. The other factor studied, Time (factor C), showed no significance at less than 95% confidence level for the responses Stability and Reusability and it was the less significant effect to the response Activity.

Table 5.3 - Estimated coefficients, standard errors and Student's *t*-test for conjugate activity (CA), operational stability at 60ºC (OS) and reusability (R_5), using the 2^4 full factorial design

Factors	CA (U/ml)			OS (%)			R_5 (%)		
	Coefficient	Standard error	*t* value	Coefficient	Standard error	*t* Value	Coefficient	Standard error	*t* value
Intercept	2.45	± 0.048	---	66.80	± 3.18	---	53.89	± 1.23	---
A: Eudragit	0.53	± 0.048	11.10[a]	7.81	± 3.18	2.46[d]	20.24	± 1.23	16.47[a]
B: Carbodiimide	-1.27	± 0.048	-26.54[a]	12.54	± 3.18	3.94[c]	7.17	± 1.23	5.83[b]
C: Time	-0.17	± 0.048	-3.50[c]	-1.49	± 3.18	-0.47	1.34	± 1.23	1.09
D: Blocking agent	-0.31	± 0.048	-6.48[b]	11.59	± 3.18	3.65[c]	4.33	± 1.23	3.52[c]
AB	-0.046	± 0.048	-0.97	8.00	± 3.18	2.52[d]	-8.41	± 1.23	-6.84[b]
AC	-0.046	± 0.048	-0.97	0.87	± 3.18	0.28	-0.51	± 1.23	-0.41
AD	-0.021	± 0.048	-0.44	3.27	± 3.18	1.03	0.081	± 1.23	0.066
BC	-0.0025	± 0.048	-0.052	-0.70	± 3.18	-0.22	-0.33	± 1.23	-0.27
BD	-0.027	± 0.048	-0.57	9.50	± 3.18	2.99[d]	3.73	± 1.23	3.04[d]
CD	0.093	± 0.048	1.93	-3.13	± 3.18	-0.98	-0.72	± 1.23	-0.58
Center point	-0.42	± 0.11	-3.95[c]	1.70	± 7.11	0.24	-5.69	± 2.75	2.75

[a] $p < 0.0001$ [b] ($0.0001 < p < 0.001$) [c] ($0.001 < p < 0.01$) [d] ($0.01 < p < 0.05$)

5.3.1 EFFECT OF PARAMETERS IN CONJUGATE ACTIVITY

Analysing the response CA (remaining activity of the prepared immobilized conjugates), it was seen that all the four main factors had statistical significance at less than 99.5% of confidence level. Nevertheless, the effect of Time on conjugate activity (p=0.0037) is considerably lower than the other effects (p<0.0001). The Eudragit concentration has a positive effect, meaning that its increase maximizes the overall response, while the other 3 effects have a negative effect, meaning that they should be decreased in order to maximize the retained activity of the conjugate. No interaction effects were significant at less than 95% confidence level, so the linear mathematical model proposed for this response, in actual terms, is:

$$CA\ (U/mL) = 4.17 + 0.53*A - 3.17*B - 0.084*C - 1.13*D \quad (5.2)$$

This model presents an R^2 of 0.98 with an adjusted R^2 of 0.98 in good agreement with the predicted R^2 (0.97) and it was significant at a confidence level less than 99.99% ($p<0.0001$).

Although this model presented curvature significant at less than 99.85% (p<0.0015), showing that the area studied should be extended to perform a correct analysis, our goal was to study the influence of these parameters on the three responses (Activity, Stability and Reusability) and to maximize them in this range, so the model was accepted, and its ANOVA table is shown in Table 5.4. Another proof of our model, as it can be seen from the ANOVA table, is that it presents no lack of fit and its significance (p<0.0001) is much higher than the curvature's probability level (p=0.0015), having also the residuals distributed along a well

randomized straight line.

Table 5.4 – Analysis of variance (ANOVA) for the representative model of conjugate activity, in the area studied

Source	SS	d.f.	MS	*F value*	*p*
Model	32.31	4	8.08	218.35	< 0.0001
Curvature	0.57	1	0.57	15.44	0.0015
Residual	0.52	14	0.037		
Lack of Fit	*0.48*	*11*	*0.044*	*3.73*	*0.1530*
Pure error	*0.035*	*3*	*0.012*		
Total	33.40	19			

R^2 = 0.98; C.V. = 8.14%

SS = sum of squares; d.f. = degrees of freedom; MS = mean square

In all the assays performed, the activity yield expressed by the conjugate was below 45% (achieved for assay 2), even though most of the protein added was coupled (no protein or enzyme activity were detected in the washings). These results are in agreement with the results previously published by other authors (Fujimura *et al*. 1987; Arasartnam *et al*. 2000). The reduction in the activity expressed could be due to either enzyme denaturation by the coupling conditions or to the intermolecular binding between the enzyme molecules and Eudragit S-100, causing steric hindrance effects. Since the activity was detected using a high molecular weight substrate (casein), the steric effects are more obvious, explaining the low conjugate activities. Arasaratnam *et al.* (2000) showed that the covalent coupling of trypsin to Eudragit S-100 resulted in pronounced steric hindrance when acting toward the high-molecular weight substrate, even when the enzyme molecules remained catalytically active. However, for low molecular weight substrates this effect was not evident.

The contour plot for the activity in the area studied (Figure 5.2) confirms the linearity of the model and clearly shows that is possible to increase the final conjugate activity by decreasing carbodiimide and increasing Eudragit concentration.

The molar ratios of Protex Multiplus L coupled to Eudragit ranged from 0.36 to 1.80 (enzyme:polymer). The lower molar ratio was attained for the maximum concentration of Eudragit (corresponding to 62 mg Protex/g Eudragit) while the molar ratio of 1.80 was attained for the lower level of Eudragit (corresponding to 308 mg Protex/g Eudragit) since the enzyme was added in a fixed amount. To these high molar ratios, crowding of the molecules on the polymer might have happened. This can partially explain the higher activities of the conjugate when Eudragit was in the maximum amount, since the enzyme:polymer ratio was smaller, meaning a higher number of multivalent interactions with polymer backbone per molecule of enzyme (Rodrigues *et al*. 2002). Dourado (Dourado *et al.* 2002) found that the clustering effect between Eudragit and cellulase existed when the molar ratio (enzyme:polymer) went beyond 1.

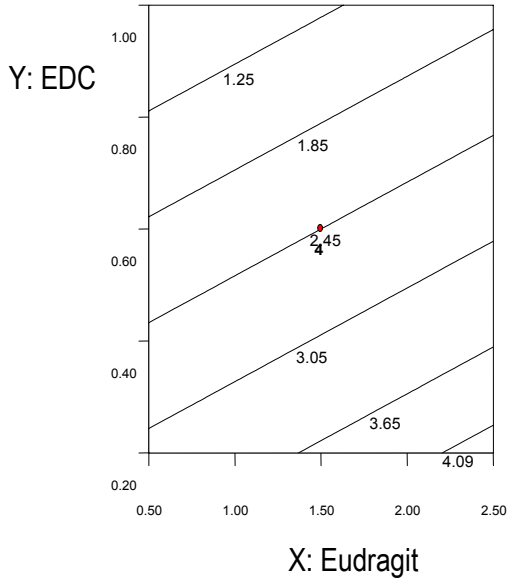


Figure 5.2 – Contour plot showing the effect of Eudragit and carbodiimide concentration on the final activity of the conjugate. The other factors were kept at the central level.

5.3.2 EFFECT OF PARAMETERS IN CONJUGATE STABILITY

For the operational stability, measured after keeping the immobilized enzymes for 5 hours at 60ºC, it was seen that the factor A and the interaction AB showed statistical significance at a confidence level of 95% and the factors B, D and the interaction BD at a confidence level of 99%. These factors were then included in the representative linear model, in actual terms, for this response:

$$OS\ (\%) = 57.42 - 4.19*A - 26.72*B - 9.68*D + 20.00*AB + 86.36*BD \qquad (5.3)$$

The statistical significance of the first-order model equation was evaluated by the *F*-test (ANOVA), which revealed that this regression is statistically significant (*p=0.0001*) at a confidence level of 99.99% (Table 5.5). In addition, the

model did not show lack of fit and had a correlation coefficient (R^2) that explains 83% of the variability in the response.

Table 5.5 – Analysis of variance (ANOVA) to the representative model of operational stability at 60ºC, in the area studied

Source	SS	d.f.	MS	*F value*	*p*
Model	8107.91	5	1621.58	12.57	0.0001
Curvature	9.25	1	9.25	0.072	0.7931
Residual	1676.49	13	128.96		
Lack of Fit	*1451.95*	*10*	*145.20*	*1.94*	*0.3191*
Pure error	*224.54*	*3*	*74.85*		
Total	9793.65	19			

R^2 = 0.83; C.V. = 16.91%

SS = sum of squares; d.f. = degrees of freedom; MS = mean square

Analyzing the contour plot obtained for this response (Figure 5.3), it is possible to see, by the linear horizontal shape of the curve, that when the parameter carbodiimide concentration is in the lower level there is no interaction effect among eudragit and carbodiimide. But when this parameter is in the upper level, the interaction effect among them becomes significant, and the higher values for the activity after 5 hours at 60°C are attained using the upper level of these two factors.

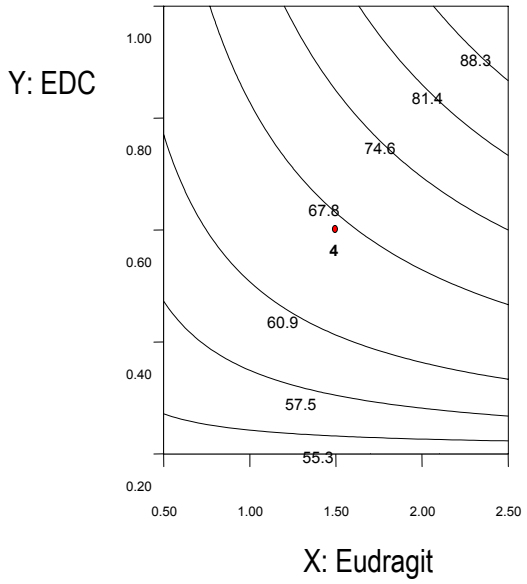


Figure 5.3 – Contour plot showing the effect of Eudragit and carbodiimide concentration on the operational stability of the conjugate at 60ºC. The other factors were kept at the central level.

The operational stability of the native enzyme after 5 hours at 60°C was only 30%. Thus, the conjugate is much more stable at this high temperature. This stabilizing effect caused by the immobilization into Eudragit polymers has been reported by several authors (Sardar *et al*. 2000; Sharma *et al*. 2003; Fujimura *et al.* 1987). Enhanced stability seems to depend on the rigid conformation of the enzyme modified by water-soluble carbodiimide and/or by covalent binding to the polymer (Fujimura *et al.* 1987). It is important to refer that in the case of proteases, immobilization is known to reduce autolysis. So, a useful outcome of immobilization is thus enhanced storage stability of the enzyme (Sharma *et al*. 2003).

5.3.3 EFFECT OF PARAMETERS IN CONJUGATE REUSABILITY

The response reusability (R_5), which measures the activity of the immobilized preparation after 5 cycles of precipitation/dissolution, was evaluated. Figure 5.4 shows that the reusability was higher when using the higher concentration of Eudragit in the immobilization procedure. When using the lower concentration of Eudragit, the reutilization factor after 5 repeated cycles of reutilization varies greatly from 60% (assay 11) to 14% (assay 1). Therefore, at the lower level of eudragit concentration a strong interaction effect between eudragit and carbodiimide exists, being the higher values for R_5 attained when carbodiimide is at its maximum level. This variation was not observed when 2.5% of Eudragit was used on the immobilization procedure. These last assays were then considered to be covalently crosslinked. It is known that non-covalently bound enzyme is easily lost in repeated reaction cycles and Arasaratnam (Arasaratnam *et al*. 2000) found that when trypsin was immobilized to Eudragit S-100 in the absence of EDC, only 2% of the activity was retained after the 3rd cycle.

The serine alkaline protease Protex Multiplus L has a pI around 9 and coupling to Eudragit S-100 was performed at pH 7.6. At this pH the protein is positively charged and the polymer has an opposite charge. It is then likely that hydrophobic interactions do not play a major role in Protex adsorption to Eudragit, favouring the covalent crosslinking (Rodrigues *et al*. 2002). Also, the immobilization procedure was performed in the presence of high salt concentrations (0.14 M NaCl) to cut down adsorption due to electrostatic interactions (Tyagi *et al*. 1998).

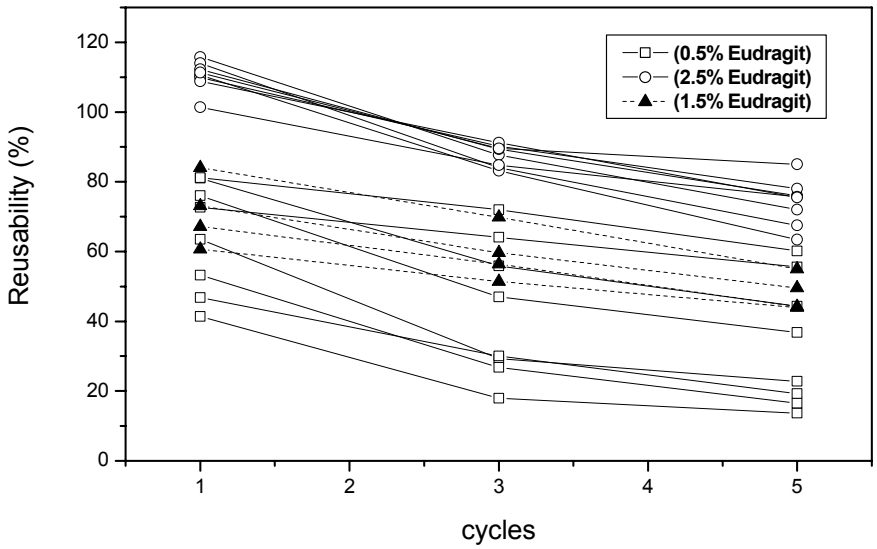


Figure 5.4 – Effect of Eudragit concentration on the remaining activity (in %) of the conjugate after several cycles of precipitation/dissolution (initial activity was taken as 100%).

The Student *t*-test confirms the interaction effect between A and B (Table 5.6). The reusability response presents therefore A, B and the interaction among them as significant factors at 99.5% confidence level. The model attained for R_5, in actual terms is:

$$R_5\ (\%) = -6.14 + 32.85*A + 49.45*B - 21.02*AB \qquad (5.4)$$

The analysis of variance (ANOVA, Table 5.6) demonstrates that the model is highly significant ($p<0.0001$) and the R^2 value, being the measure of the goodness of the fit, indicates that 92% of the total variation is explained by the model. It presents no curvature and no lack of fit as significant factors.

Table 5.6 – Analysis of variance (ANOVA) to the representative model of reusability, in the range studied

Source	SS	d.f.	MS	*F value*	*p*
Model	8509.85	3	2836.62	56.03	< 0.0001
Curvature	103.74	1	103.74	2.05	0.1728
Residual	759.36	15	50.62		
Lack of Fit	*677.52*	*12*	*56.46*	*2.07*	*0.2999*
Pure error	*81.84*	*3*	*27.28*		
Total	9372.95	19			

R^2 = 0.92; C.V. = 13.49%

SS = sum of squares; d.f. = degrees of freedom; MS = mean square

The contour plot attained for reusability (Figure 5.5) confirms the existence of an interaction effect at the lower levels of A and B, while at the upper level of Eudragit, the concentration of carbodiimide is not affecting this response.

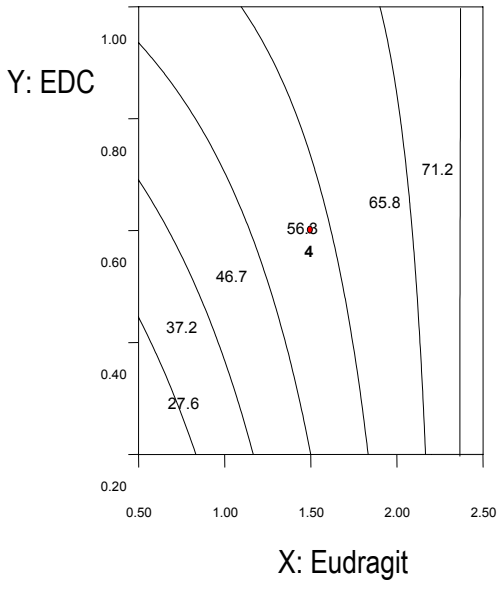


Figure 5.5 – Contour plot showing the effect of Eudragit and carbodiimide concentration on the reusability of the conjugate. The other factors were kept at the central level.

Since it was intended to optimize the immobilization procedure in order to maximize all the analyzed responses, the graphical optimization of the statistical program Design-expert was performed. The method basically consists of overlaying the curves of the models according to the criteria imposed (Silva and Roberto 2001). Based on the three models obtained, a graphical optimization was conducted using a statistical program (Design-expert), defining the optimal working conditions to attain high conjugate activity, operational stability at 60°C and reusability. The criteria imposed to the enzyme conjugates were: (a) the activity should be no less than 3 U/mL, (b) the operational stability at 60°C should be more than 50% and (c) the reusability after the 5th cycle should be above 65%.

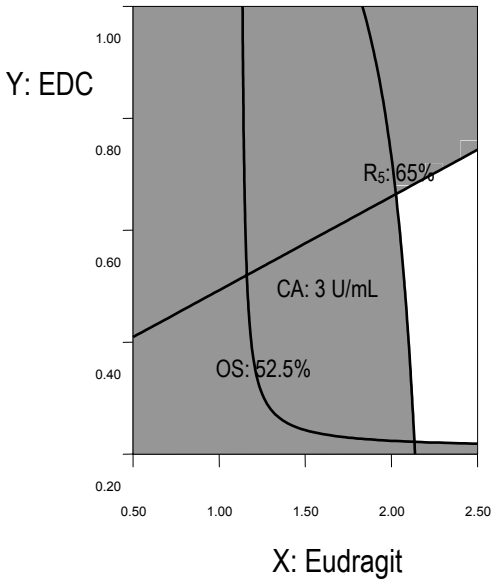


Figure 5.6 – The optimum region by overlay plots of the three responses evaluated (activity, operational stability and reusability) as a function of eudragit and carbodiimide concentration. The other factors were kept at the lower level.

The overlay plot attained (Figure 5.6) shows a non-shaded area where all these criteria are satisfied. The time of coupling and the blocking agent

concentration were kept at its lower levels (C=1 hour and D=0.05% v/v) by economical reasons, since they exerted less statistical influence in the responses tested than the Eudragit or carbodiimide concentrations.

Thus, a point was chosen on the graph, which was assigned as optimum point corresponding to 2.5% (w/v) of Eudragit (coded level +1) and 0.2% (w/v) of carbodiimide (coded level -1). As previously stated, for economical reasons, the time and blocking agent were set at their lower values. These settings were the same described by assay 2 of the experimental statistical design. Under these conditions, the models attained predict the following values for the responses, together with the experimental error in the 95% confidence interval:

Conjugate Activity (CA): 4.7 U/mL [4.50 – 4.96]

Operational Stability (OS): 52 % [36.97 – 67.01]

Reusability (R_5): 75 % [67.79 – 82.96]

The values attained in assay 2 are in good agreement with the predicted values for the analyzed responses, validating the mathematical linear models attained.

Regarding the native enzyme, used as control, the following parameters were attained for the immobilization of the commercial alkaline serine protease Protex Multiplus L to Eudragit S-100 in the optimum conditions defined:

Table 5.7 – Coupling of Protex Multiplus L to Eudragit S-100, after activation by carbodiimide[a]

Sample	Protein, mg/ml	Activity, U/ml	Specific Activity, U/mg	$\eta_{protein}$[b], %	$\eta_{activity}$[b], %
Native enzyme	0.77	10.35	13.44	100	100
Eudragit-Protex conjugate	0.56[c]	4.70	8.39	73	45

[a] each experiment was done in duplicate. The difference in the individual readings was less than 5%.

[b] $\eta_{Protein}$ is the protein coupling yield and $\eta_{activity}$ is the activity yield of Eudragit-Protex conjugate.

[c] protein measured directly on the conjugate after re-dissolving using a solution of Eudragit S-100 that followed the same protocol without adding protease as a blank.

The specific activity was lower after immobilization, confirming the existence of steric hindrance effects. This was expected since multivalent interactions between the enzyme molecule and the polymer after coupling may induce structural rearrangements on the protein molecule. This steric crowding phenomenon has been observed in many earlier studies (Arasaratnam *et al*. 2000; Dourado *et al.* 2002; Sardar *et al*. 2000).

Interestingly, the specific activity was always higher to the lower levels of carbodiimide used in the coupling procedure (see Figure 5.7). This result is reinforcing the idea that a clustering effect is occurring: when more carbodiimide is added, more intermolecular cross links are formed, which may avoid the approach of the macromolecular substrate to the enzyme active site. This phenomenon is likely to be more pronounced with a macromolecular substrate like casein.

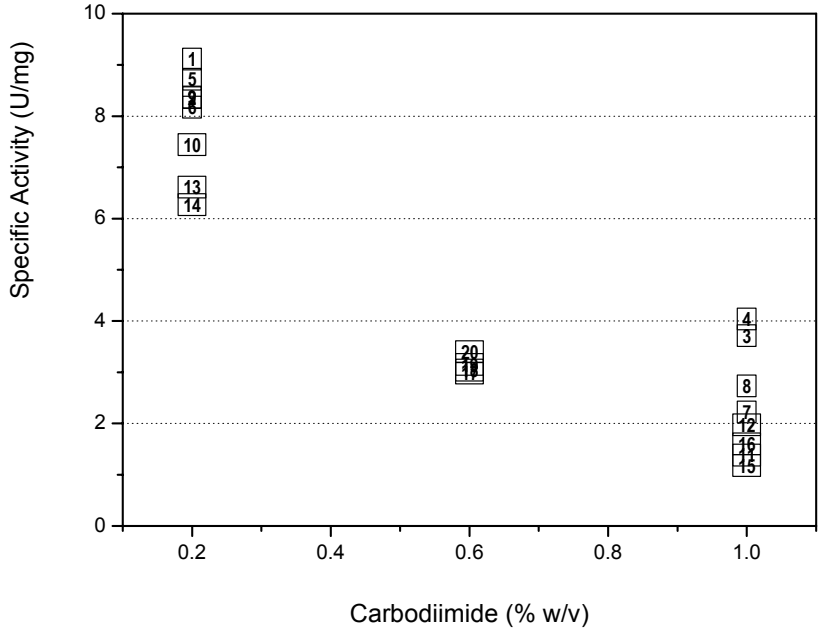


Figure 5.7 – Effect of carbodiimide concentration (% w/v) on the specific activity of the conjugate. The numbers refer to the number of the assay in the statistical standard order (Table 5.2).

5.4 CONCLUSIONS

Information about several parameters that influence the immobilization of proteases into reversibly soluble/insoluble polymers and their interactions can be obtained by the factorial methodology, requiring a limited number of experiments, when compared with classical methods. Using the methodology of experimental factorial design it was possible to determine optimum coupling conditions for a serine protease to Eudragit S-100 and obtain a very stable covalently crosslinked conjugate with high activity and reusability. This optimized conjugate could be attained using lower concentrations of the coupling and blocking agents and less

time of coupling, meaning economical advantages over the previous coupling procedure. More importantly, the non-specific adsorption was eliminated, which represents an improvement in the carbodiimide coupling to Eudragit S-100.

All of the four factors studied have played a critical role in the protease coupling. Response surface methodology was used as an optimisation strategy to attain a conjugate with high activity yield and operational stability at 60°C. Under optimised conditions (Eudragit, 2.5% w/v, carbodiimide, 0.2% w/v, coupling time,1 h and blocking agent concentration, 0.05%), the conjugate activity yield was about 45% and its operational stability at 60°C increased of 1.7 times. After reusing the conjugate for 5 cycles, the remaining activity was still 72% of the initial value when compared to the native enzyme. Several tests confirmed that the enzyme was covalently crosslinked to eudragit.

This stable biocatalyst can be used for the hydrolysis of macromolecular or insoluble substrates, since it is reversibly soluble-insoluble, eliminating the mass transfer limitations in heterogeneous systems. In addition, the convenient handling of the enzyme preparations, the easy separation of the enzyme from the product and the re-use of the enzyme provides a number of cost advantages, which are often an essential pre-requisite for establishing an economically viable enzyme-catalyzed process.

CHAPTER 6

GENERAL DISCUSSION

"The whole of science is nothing more

than a refinement of everyday thinking."

Albert Einstein (1879-1955)

German physicist

6. General Discussion

Total easy care is a concept that wool finishers in particular must address and solve over the next few years. Total easy care must confer ease of maintenance and impart high performance to wool garments. In the future the necessity to obtain total easy care wool i.e. machine washability plus tumble dryability, to compete with other fibres will become more pressing. The most commonly used method to confer dimensional stability to wool articles is the chlorine-Hercosett, which has various drawbacks as previously reported. Several enzymatic methods have been attempted to replace this hazardous chemical finishing treatment, without great success.

Our work also used proteolytic enzymes, but modified ones, in order to avoid their penetration inside the fibre, which is the major obstacle to the implementation of an enzyme finishing process, due to the high undesirable weight loss caused.

The work done and presented in this thesis is one small step towards the study and implementation of an environmental friendly process for wool bio-finishing using proteases. Each chapter presents the discussion of the respective results and conclusions. However, there are some aspects that deserve to be pointed out, since that only now is it possible to make an overall analysis of the work.

First of all, it was necessary to prove the concept underlying this thesis. Due to the fact that the immobilization of enzymes by covalent coupling is often accompanied by steric hindrance effects, the investigated approach was uncertain.

The fact that the proteases were bigger in size could implicate steric hindrance effects of such a magnitude that the proteases would not be able to interact with wool, hydrolysing its cuticle. Thus, chapter 2 intended to be a starting point for our work, proving that the concept could work properly. In the study described in that chapter, two commercial proteases were acquired, with different molecular weights and their hydrolytic behaviour towards wool fibres was analysed and compared. Native subtilisin (with average M*r* close to 20 kDa) and subtilisin-PEG (with average M*r* close to 110 kDa) were used to study the adsorption into wool fibres, after different pre-treatments. It was observed that:

- The penetration of protein (measured as the maximum adsorption capacity in g protein/g wool) was higher when the wool was previously subjected to a surfactant washing and bleaching. It is believed that this treatment is more effective in removing the bounded fatty acid barrier of the epicuticle. Thus, as already pointed out by several authors, the proteolytic treatment is enhanced by wool pre-treatments such as the surfactant washing and bleaching.
- Secondly, it was verified that subtilisin-PEG showed little absorption (only about 7%) while native subtilisin showed 50% adsorption into wool. Also, native subtilisin produced a high amount of released aminoacids (indicating wool fibre degradation) and its tensile strength was lower, comparing with subtilisin-PEG.
- Furthermore, by using fluorescence techniques, it was possible to follow the diffusion of the proteases inside wool and conclude that their diffusion was dependent on their size. The native subtilisin penetrated completely into wool fibre cortex while the modified enzyme, with a bigger size, was

retained at the surface, in the cuticle layer. It was also observed that proteins without proteolytic action do not adsorb on wool, due to its hydrophobic nature.

This chapter allowed for an important conclusion: it is possible to control the hydrolysis process on wool, by using enzymes with an adequate size and thus retaining activity on the wool surface.

The subsequent chapters (3 and 4) present some of the techniques attempted to modify the proteases in order to achieve the desired results. The modifications performed were mainly by covalent coupling the enzymes to several matrices, thus increasing their size. Since wool is a solid substrate, the interaction of wool with the enzyme would only occur if the enzyme was in the soluble state. So, the need for soluble conjugates was a priority.

The first attempt (chapter 3) was by coupling the proteases to a known and extensively studied bifunctional reagent, glutaraldehyde. This bifunctional reagent proved to be very effective in crosslinking proteins possessing a high amount of Lysine residues, which are usually accessible for reaction since they tend to be at the exterior of the protein.

- It was detected that the M*r* of the proteins was gradual increasing with the increase of final glutaraldehyde concentration and that its addition should be done slowly and in small amounts.
- The method of covalent crosslinking using the bifunctional reagent glutaraldehyde was not effective for increasing the proteases molecular weight, either due to the low amount of free lysine residues available in the studied protease for crosslinking or to the presence of other amines in the commercial preparation that might have reacted preferentially with this

compound.

Several other approaches for creating soluble enzymatic conjugates were attempted (described in the international patent – Appendix A), using soluble polymers, like PVA or PEG. The covalent coupling of the proteases to soluble-insoluble polymers was studied as the enzymatic conjugate would be much more interesting from an industrial point of view if one could recover the enzyme after the treatment, allowing for reuse and continuous operation.

Thus, chapter 4 describes the covalent coupling of Esperase, a commercial protease to a soluble-insoluble polymer of high molecular weight (Eudragit S-100). This conjugate exhibited the following characteristics:

- When comparing to the native enzyme, the immobilized form presented a lower specific activity towards high molecular weight substrates, but a higher thermal stability at all temperatures tested. It also exhibited a good storage stability and reusability, which makes this enzyme conjugate quite interesting from an industrial point of view.
- Wool fabrics treated with the immobilized serine protease using harsh conditions and subjected to several machine washings, presented a significantly lower weight loss than wool treated with the native enzyme, in the same conditions. Using a moderate enzymatic treatment, a reduction to about half of the initial area shrinkage was attained, both for free and immobilized enzymes. However, wool fabric treated with the immobilized protease presented 92% of the original tensile strength resistance while that treated with native enzyme kept only 75% of its initial resistance.
- An enzyme conjugated to such a carrier may be used as a catalyst in its

soluble form and then be recovered via the insoluble state, overcoming the problem of the non accessibility of the enzyme to the macromolecular substrate, wool. It also eliminates the need for an enzyme-denaturising step in any enzymatic wool-processing with this enzyme. It is known that adsorbed proteases are not easily rinsed from treated wool fibres and that the enzyme retained in the fibre continues to catalyse hydrolysis of the protein substrate under normal storage or use conditions (Nolte 1996). Thus, this immobilization method ensures removal of all the covalently linked protease, protecting the fibre from progressive deterioration.

Although this method proved to be very effective for wool finishing, the presence of native enzyme in the conjugate was a drawback. Thus, the immobilization method needed to be optimized, in order to understand the effects of each variable in the coupling procedure and of their interactions, and to remove the non-covalently bounded enzyme. Chapter 5 relates the optimization of the coupling of the protease to the polymer, Eudragit S-100, by using experimental design techniques. This optimization strategy allowed for an enzyme conjugate wherein the enzyme was covalently cross linked to the polymer, with high activity yield and high operational stability at 60ºC. These statistical techniques proved to be useful in understanding the effects of several variables affecting a process and of their interactions, while allowing for optimization with few assays.

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

"The future belongs to those who believe

in the beauty of their dreams."

Eleanor Roosevelt (1884-1962)

Mrs. Franklin D. Roosevelt

7.1 CONCLUSIONS

The results presented in this thesis prove that modified proteases attained by the described immobilization method, using a soluble-insoluble polymer of high molecular weight, can be a promising alternative for wool bio-finishing processes at an industrial level, since it is an effective way of removing wool scales and can be an environmental friendly option to the conventional chlorine treatments. This process needs to be further characterized for its complete understanding and optimization.

Therefore, the innovative aspects of this thesis are:

- the feasibility of controlling the hydrolytic action of the enzymatic treatment by the use of an immobilized protease;

- the possibility of recovering the enzyme for posterior treatments which allows for important cost savings in industrial implementation;

- the development of a promising environmental friendly alternative to the conventional chlorine-Hercosett shrink-resist treatment of wool.

7.2 FUTURE PERSPECTIVES

Further studies will contribute to the understanding of the mechanism of the proteolytic attack and will completely elucidate the factors that affect this reaction, like the smallest proteases molecular weight needed to avoid their penetration inside the wool fibre. Thus, it is our intent to:

- Continue this study, upgrading the protease modification technology from laboratory to a large-scale process, allowing for a new green industrial process to be developed and implemented for the enzymatic treatment of protein fibres, which would represent a major technological breakthrough.
- Find the optimal process parameters for the anti-felting treatment of wool, where weight loss, shrinkage and tensile strength will be minimized.
- Develop new formulations for industrial and domestic wool carpet cleaning and garment washing. The stability of the soluble-insoluble enzymatic conjugates on detergents (compatibility with detergents components as surfactants, perfumes and bleaches), their performance on cleaning stains and the possible damages inflicted on wool in domestic washing will be investigated.
- Produce new modified proteases with reduced diffusing ability in wool. It is our intent to test other soluble-insoluble polymers available for immobilization, like the PNIPAAm (poly(N-isopropylacrylamide), a thermo-responsive polymer, and also to test the potentialities of engineered proteases with high molecular weight. It is desirable that these enzyme conjugates be separated by molecular weight fractions, in order to evaluate and better understand the potential of the several conjugates on wool bio-

finishing.

- Develop bio-scouring processes (using an enzymatic cocktail of cellulases, pectinases, lipases, xylanases, hemicellulases and this modified protease) to achieve significant improvements in scouring efficiency, whiteness and dyeability of wool, which would reduce the chemical use and costs of the conventional scouring method.
- Develop low temperature dyeing processes for wool fibres by pre-treatment with the new modified protease.

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"Nothing in this world is to be feared…

only understood."

Marie Curie (1867–1934)

Nobel Prize in Physics (1903) and Chemistry (1911)

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APPENDIX A

TREATMENT OF ANIMAL HAIR FIBERS WITH MODIFIED PROTEASES

"Science knows no country, because knowledge belongs to humanity,

and is the torch which illuminates the world."

Louis Pasteur (1822-1895)

French chemist biologist,

and founder of microbiology

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date:
27 November 2003 (27.11.2003)

PCT

(10) International Publication Number
WO 03/097927 A1

(51) International Patent Classification⁷: D06M 16/00, C12N 11/08

(21) International Application Number: PCT/IB02/00808

(22) International Filing Date: 21 May 2002 (21.05.2002)

(25) Filing Language: English

(26) Publication Language: English

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regionally*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/097927 A1

(54) Title: TREATMENT OF ANIMAL HAIR FIBERS WITH MODIFIED PROTEASES

(57) Abstract: A method of treating fibers of animal origin wool from sheep, cashmere, rabbit, mohair, llama, goat, camel, among others), characterised in that it consists of bringing the fiber in contact with a solution of modified proteases, treated or not with other substances, in order to increase its molecular weight and reduce its diffusion inside the fiber. It is intended that the cuticle of the fiber be the only accessible part to the protease attack. It has shown an increase in the resistance to shrinkage tests and felt finishing, in comparison with untreated material.

DESCRIPTION

" TREATMENT OF ANIMAL HAIR FIBERS WITH MODIFIED PROTEASES "

Field of the invention

The cuticle layer of animal hair fibers presents a scale structure when observed by microscopy. The felting or shrinkage of these fabrics is due to the overlapping of these scales that surround the cortex (inner part of the fiber), in wet processes with high mechanical agitation. The removal of the cuticle layer allows for elimination of the tendency of the protein fibers of animal origin to shrink. One possibility of anti-felt treatment would be the application of proteolytic treatments for the removal of the cuticle layer. This kind of treatment has been extensively studied since the beginning of the XX century, but without great achievements.

The reasons for this are primarily due to the following factors:

- The hair fibers of animal origin have a very variable composition, which depends on origin, race, climate and animal feeding. This diversity of animal fibers induces several susceptibilities to the proteolytic treatments.

- Higher aggressive treatments to induce a uniform anti-felting behaviour to all the fibers, cause by consequence unacceptable strength loss.

- Recent studies indicate that the lack of reproducibility of the proteolytic treatments and the degradations caused by such treatments are due to the diffusion of the enzymes inside the animal fibers.

Background to the invention

The most commonly used method to confer dimensional stability to articles made from animal hair fibers is the INS/CSIRO Chlorine/Hercosett, which comprises a strong acid chlorine treatment, followed by the application of a polymer resin. This process results in an increased degree of shrinking resistance, but has a number of drawbacks: poor handle, limited durability, difficulties in dyeing and, more importantly today, it generates environmentally damaging waste.

Several authors have suggested some methods to reduce the shrinkage of animal fibers, like wool, for instance, which do not result in release of damaging substances to the environment. Among such processes, there are the enzymatic ones, as well as benign chemical processes such as low-temperature plasma treatments. Plasma treatment is a dry process, which involves treating wool fiber material with electric gas discharges (so-called plasma). At present, there are obstacles (costs, compatibility and capacity) to large-scale commercialisation of a plasma treatment process.

Several enzymatic methods have been used on wool treatments. The patent JP-A 51099196 describes a process to treat wool fabrics with alkaline proteases. The patent JP-A 3213574 describes a method for the treatment of wool with transglutaminase or a solution having this enzyme. The patent US 6051033 describes a method of wool or wool fibers treatment with a proteolytic enzyme and tranglutaminase. WO 98/27264 describes a method to reduce the shrinking of wool that comprises the contact of fiber samples with a solution of peroxidase or oxidase under the adequate conditions for the enzymatic reaction with wool. The patent US 6099588 relates a method to improve the shrink resistance that may result in improvements in handle, appearance and felting, among others, by the application of proteolytic enzymes in an aqueous solution, after a treatment with an alkaline solution having alcohol.

The patent US 5.529.928 refers to a process to attain wool with anti-felt finishing, soft handle and with shrink resistance using an initial chemical oxidation followed by a treatment with protease and warming. The patent EP 134267 uses a similar process, treating the fiber with proteolytic enzymes in the presence of salt, after the initial oxidative treatment. The patent EP 3.58386 describes a method of wool treatment that comprises a proteolytic treatment and one of, or both, an oxidative treatment (such as NaOCl) and treatment with polymer.

The necessity of establishing environmentally friendly (Eco-friendly) methods with better performances than the industrial processes currently used, drives the need to create new processes that give a good shrink resistance, softness, appearance and anti-pilling behaviour. Therefore, a new methodology of enzymatic treatment of animal hair fibers is presented here.

Summary of the invention

This invention relates to a new enzymatic process of animal hair fiber treatment, in which the proteases are chemically modified in order to increase their molecular weight and therefore reduce their diffusion inside the fiber. The cuticle will be the only accessible part to the proteolytic attack, which allows for the improvement of one or more wool properties, including their felting and shrinking, without damaging the fiber's interior.

The methodologies used to increase the molecular weight of the enzymes are based on the utilisation of a soluble polymer with hydroxyl groups or others (carboxylic groups), activated with γ–aminopropiltrietoxisylane, carbodiimide and/or glutaraldehyde. The glutaraldehyde molecules may link afterwards, covalently, to another polymer chain, forming a polymeric net, or to an available protein NH_2 group.

Detailed description and Examples

The method consists in the treatment of the proteic material with a solution of modified proteolytic enzymes. Commercially available proteases from Sigma and Genencor (*Subtilisin* kind) were used.

Immobilisation was performed using soluble and/or soluble-insoluble polymers. Polyvinyl alcohol (Sigma), of medium average molecular weight 70000–100000, polyethylenglycol (Sigma) of 10000 of average molecular weight and a copolymer of methacrylic acid-methyl methacrylate of 135000 of average molecular weight, were used. As coupling agents, glutaraldehyde (Aldrich), γ–aminopropiltrietoxisylane, carbodiimide and/or Borax (Sigma) were used.

The polymer at 6% (w/v) solution in distilled water was dissolved with

warming and stirring, activated, and then was added to a 2% (v/v) glutaraldehyde solution. This solution was kept under stirring at room temperature, for 2 hours. After this time, the solution was dialysed in 0.1 M pH 5.0 acetate buffer for 24 hours and then in 0.05 M pH 3.95 acetate buffer for 20 hours.

The enzymatic preparation in the desired concentration was added to the resulting solution, together with PEG (1.25%) and Borax (0.05 μg/mL) on 0.1 M pH 5.0 acetate buffer, and left under stirring for 8 hours at room temperature. This solution was kept at 4ºC until use. The immobilisation procedure did not bring any significant activity lost.

The soluble-insoluble polymer at 2% (w/v) solution in distilled water was dissolved with pH control and stirring, activated with carbodiimide, and then was added to a 2% (v/v) protease solution. This solution was kept under stirring at room temperature for 3 hours. After this time, the polymer-enzyme solution was washed by performing several cycles of precipitation-centrifugation, and was finally dissolved in 0.3 M Tris-Cl buffer pH 7.6. This solution was kept at 4ºC until use. It was verified that the immobilisation procedure brought a deviation of pH optima to higher alkaline values, while temperature profile was not affected.

Description of Preferred Embodiments:

The following examples illustrate the invention.

Example 1:

Treatment of pure wool fabric with proteases:

Samples of pure merino wool fabric (like animal hair fiber) with about 12 cm x 12 cm (of about 3 grams, each), were placed in a recipient with a solution

of chemically modified proteases, or not, using a bath relation of 1/20 (w/v). The treatment was performed at 37ºC, for periods of time ranging from 4 to 48 hours. Samples were removed from the solution, washed and air-dried. They were then subjected to tests to evaluate eventual damage caused during the treatment.

To evaluate the quality of the fabric and the degree of damage caused in the wool treatment process, a qualitative test based on Garner (Garner W., *Textile Laboratory Manual*, vol. 5 - Fibres, 3rd Edition, 1967) was used. It was verified that the modified proteases did not induce fiber degradation when compared with free proteases. The control treatment itself (10 mM pH 7.5 acetate buffer) presents a level of fiber degradation superior to that presented by the fibers treated with modified enzymes.

The tendency of the fabrics to shrink was verified by washing the fabrics (11 x 6 cm) three times in distilled water having 50 μL of a wetting agent for 60 minutes, at 50ºC and 20 rpm. The shrinkage was measured by the variation of the specimen dimensions. It was verified that only the fabrics enzymatically treated did not induce a significant shrinkage.

A panel of 5 technicians evaluated the handle and appearance of the wool fabric, verifying an increase in the properties of the protease treated fabrics relatively to the control fabric.

Example 2:

Treatment of pure wool yarns with proteases:

Similar studies were conducted in yarns of merino wool using the following parameters: samples of pure wool yarn were placed in a recipient with a chemically modified proteases solution, or not, in a bath ratio of 1/20 (w/v). The

treatment was conducted at 37ºC, for periods of time ranging from 4 to 48 hours. The samples were removed from solution, washed and air-dried. They were then subjected to test to evaluate eventual damage caused during treatment.

To evaluate the yarn quality and the degree of damage caused in the treatment process of this fiber, a qualitative test based on Garner (Garner W., *Textile Laboratory Manual*, vol. 5 - Fibres, 3rd Edition, 1967) was used. It was verified that the modified protease treatment does not induce degradation when compared with free protease treatment. The control treatment (10 mM pH 7.5 acetate buffer) presented a superior degradation level than that presented by the fibers treated with the modified enzymes.

Tensile strength tests were performed on wool yarns, and it was verified that only the yarns treated with free proteases induced a significant strength loss.

The tendency to shrink was verified by repeated washing of wool yarns, three times in distilled water having 50 μL of a wetting agent for 60 minutes, at 50ºC and 20 rpm. Shrinkage was quantified by the visual verification of yarn felting. It was verified that only enzymatically treated yarns did not induce felting.

A panel of 5 technicians evaluated the yarns appearance and verified a better look in the yarns treated with proteases, compared to the control yarns.

Example 3:

Treatment of pure wool fabrics with soluble-insoluble proteases:

Samples of pure merino wool fabric (like animal hair fiber) with about 15 cm x 15 cm and with about 8 cm x 20 cm, were placed in a recipient with a

solution of chemically modified proteases, or not, using a bath relation of 1/20 (w/v). The treatment was performed at 37ºC, for periods of time ranging from 12 to 72 hours. Samples were removed from the solution, washed and air-dried. They were then subjected to tests to evaluate eventual damages caused by treatment.

The storage stability of enzyme preparations was evaluated and it was verified that room temperature storage of these enzymes was possible, with no lost of activity for more than 3 months. An increase of half-life time was observed in the immobilised enzymes at all the conditions tested (4º, 20º, 37º and 60ºC).

Tensile strength tests were performed on wool rectangular fabrics, and it was verified that the fabrics treated with free proteases induced a significant strength loss (keeping only 30-40% of its initial resistance) while that treated with the immobilised proteases kept about 80% of its resistance.

The fabrics weight loss was also evaluated and for the free protease treatment it was very high, reaching 36% when wool was pre-treated prior to enzymatic treatment and was only 5% with the immobilised protease in the same conditions.

The tendency to shrink was verified by repeated washing (three times) of wool fabrics, in a domestic washing machine, at 40ºC and with 2 g/L of a commercial detergent, for approximately 50 minutes. The shrinkage was measured by the variation of the specimen dimensions. It was verified that the fabrics treated with free enzyme presented a significant shrinkage (14%), comparing to that presented by the fabrics treated with the immobilised enzymes (6%).

The fiber damage was also evaluated by SEM microscopy, where it was possible to verify that the treatment with the immobilised enzymes seems to

damage just the cortex layer of the yarn, or to a less extent, the whole wool fiber.

CLAIMS

1. A method of treating fibers of animal origin (wool from sheep, cashmere, rabbit, mohair, llama, goat, camel, among others) comprising the contact of fiber with a solution of modified proteases linked, or not, to other substances, in order to increase its molecular weight and reduce its diffusion inside the fiber. The intention being that the cuticle of the fiber be the only accessible part to the proteases attack, thus allowing an increase of the resistance to shrinkage and anti-felt finishing, in comparison with the untreated material.

2. The method, accordingly to claim 1, characterised by comprising the treatment of the fibers simultaneously with a proteolytic enzyme and transglutaminase.

3. A method, as defined in claim 1, wherein said proteolytic enzyme is of bacterial origin.

4. The method of claim 1, wherein the proteolytic enzyme is a serine protease.

5. A method, as defined in claim 4, wherein the serine protease is a *Subtilisin*.

6. The method of claim 1, wherein the amount of protease used per kg of wool,

fiber, or hair, is in the range of 1 to 1000 g.

7. The method of claim 1, wherein the transglutaminase is derived from Streptoverticillium sp.

8. The method of claim 1, wherein a treatment bath with recoverable and reusable proteases solution is used, thus lowering the costs of treatment and the effluent production, with concomitant savings in water consumption.

9. The method of claim 1, wherein soluble polymers in aqueous solutions are used, like polyvinyl alcohol (and/or polymers with hydroxyl groups), as support in the chemical modification of proteases, without restrictions.

10. The method of claim 1, wherein soluble-insoluble polymers in aqueous solutions are used, like methacrylic acid-methyl methacrylate copolymers (and/or polymers with carboxyl groups), as support in the chemical modification of proteases, without restrictions.

11. The method of claim 1, wherein the reusable proteases in aqueous solutions are used as cleaning additives to treat wool material, like carpets and rugs, without restrictions.