Enzyme adsorption at solid-liquid interfaces

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Co-promotor: Dr. W. Norde, wetenschappelijk hoofdmedewerker in de fysische en kolloïdchemie.

S. Duinhoven

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STELLINGEN

behorende bij het proefschrift

Enzyme adsorption at solid-liquid interfaces

van

Susan Duinhoven

Opgedragen aan

mijn ouders

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VOORWOORD

Het onderzoek dat in deze dissertatie wordt beschreven, is uitgevoerd in het Unilever Research Laboratorium te Vlaardingen, binnen de sectie Fysische Chemie onder leiding van Dr. W.G.M. Agterof.

Mijn promotieonderzoek was gericht op de werking van enzymen die in wasmiddelen worden gebruikt. Het is een leerzame ervaring geweest te zien, welk uitgebreid onderzoek ten grondslag ligt aan de productie en verbetering van alledaagse consumentenproducten als wasmiddelen, margarines, mayonaises en cosmetica. Hoe vanzelfsprekend deze producten in het dagelijkse gebruik ook lijken, vanuit natuurwetenschappelijk oogpunt zijn ze zeer complex. Vele van deze systemen bevatten tientallen componenten, die in interactie met elkaar functioneren onder steeds wisselende omstandigheden.

Gaarne maak ik van de gelegenheid gebruik de directie van het Unilever Research Laboratorium te bedanken voor de geboden mogelijkheid mij binnen deze stimulerende omgeving aan onderzoek te wijden. Vier jaar lang heb ik alle ondersteuning gekregen die nodig was voor de succesvolle afronding van mijn promotieonderzoek. Zo kon ik beschikken over zes Savinase-varianten, die ter beschikking werden gesteld door Novo-Nordisk, Denemarken en het Unilever Research Laboratorium Vlaardingen. Ik heb veel te danken aan de intensieve samenwerking met de verschillende afdelingen van de groep 'Detergents' o.l.v. Ir. H. Vonkeman, de groep 'Bio- and Nutrition Sciences' o.l.v. Dr. O. Korver, en de groep 'Physical and Analytical Sciences' o.l.v. Dr. L. de Galan.

In het bijzonder heb ik gesteund op de experimentele kennis en vaardigheden van Ruud Poort en Gerrit van der Voet. Samen werkend hebben wij ons telkens weer verbaasd over de wondere wereld van de enzymen. Jullie interesse en volharding bij alle 'ups and downs' zijn voor mij een stimulans geweest. Ik wil jullie beiden bedanken voor je wezenlijke bijdrage aan de totstandkoming van dit proefschrift.

Wim Agterof dank ik voor zijn niet aflatende ondersteuning van het project. Zijn brede onderzoekservaring en zijn aandacht voor de kwaliteit van industriële research garandeerden een gunstig werkklimaat. Zijn vertrouwen in de goede afloop van de onderneming heeft mij tegen menige berg op geholpen. Er was altijd tijd en aandacht voor overleg, zowel over detailproblemen als over de grote lijnen. Ik bewaar goede herinneringen aan onze uitvoerige, vaak verhitte discussies.

Hans Lyklema, mijn promotor, wil ik bedanken voor zijn accurate en efficiënte begeleiding. Zijn aanstekelijk enthousiasme voor wetenschappelijk onderzoek heeft mij geïnspireerd. Door zijn overzicht van het vakgebied en zijn vermogen het wezenlijke van de details te scheiden, heeft hij mijn onderzoek, ook bij tegenvallende resultaten, steeds met vaste hand op koers gehouden. Van meet af aan heeft hij benadrukt, dat de complexe problematiek allereerst vanuit modelsystemen moet worden benaderd, alvorens tot praktijkproeven over te gaan. De loop van het onderzoek heeft hem ten volle gelijk gegeven.

Veel dank ben ik ook verschuldigd aan mijn co-promotor, Willem Norde. Ik heb zeer geprofiteerd van zijn grote experimentele ervaring op het gebied van eiwitadsorptie. Hij heeft me op een cruciaal moment methode en materialen kunnen leveren, die het onderzoek over een dood punt hebben heengetrokken.

De wetenschappelijke achtergrondstudie ten aanzien van consumentenproducten doorloopt het hele spectrum van de natuurwetenschappen, waardoor het onderzoek in een industrieel laboratorium multidisciplinair van aard is. Wie in zo'n omgeving werkt, kan veelzijdig onderzoek verrichten en daarbij profiteren van de grote diversiteit aan specialismen, die in het laboratorium aanwezig is. Gaarne noem ik degenen die vanuit hun specifieke deskundigheid hebben bijgedragen aan het ontstaan en de uitwerking van dit proefschrift.

Met Maarten Egmond heb ik voor mij waardevolle discussies gevoerd over enzymen en eiwitten. Peter Ravestein en Willem Antheunisse dank ik de zuivering van alle benodigde enzympreparaten. Kest Hissink, Cora Engelen en Jan Bus hebben zich ingespannen te komen tot een goede labelingsmethode voor het Savinase. De berekeningen van de electrostatische eigenschappen van de Savinase-varianten zijn uitgevoerd door Hans Peters, Dennis de Jong en Jacob de Vlieg.

Hans Meder en Petra Weisenborn hebben de CD-spectra opgenomen. Arend-Jan Adriaanse heeft tijdens zijn stage in de sectie Fysische Chemie de protease-varianten gekarakteriseerd met behulp van HPLC-scheidingstechnieken. De omslag van mijn dissertatie is ontworpen door Mari van der Giessen. I would also like to thank Jonathan Warr and Pete Hall for removing the 'steenkolen' English phrases. Any errors still occurring in the text are due to alterations subsequently made.

Hen allen dank ik hartelijk voor hun bijdragen.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Enzymes at interfaces

Enzymes are proteins with the capacity of catalysing various reactions. These biocatalysts are frequently studied as they are essential for many of the complex reactions governing life in every creature on earth. More than 10,000 enzymes are known nowadays and this number increases rapidly as the technology for separation, purification and production of enzymes advances [1].

Throughout the ages enzymes have also been used in industrial processes such as leather tanning, cheese making and the leavening of bread [2]. These processes involved enzymes in the form of animal and plant tissues or whole microorganisms. The commercial application of enzymes started at the end of the last century after the discovery of extracellular enzymes, that are enzymes excreted by the cells that produce them. The production and purification of such enzymes is much easier than that of their intracellular counterparts. Nowadays, the interest in the industrial application of a variety of enzymes is increased by the fact that these catalysts are very specific, renewable, biodegradable and that they can be produced efficiently.

Most of the enzymes produced industrially have been applied as an additive to detergent powders and liquids for household and industrial laundry washing [3,4,5]. Since 1959 bacterial alkaline proteases have been added to fabric washing detergent systems to improve the removal of protein containing stains. In general, proteases catalyse, among other reactions, the hydrolysis of peptide bonds in proteins. Several "families" of proteases can be distinguished with respect to their pH optimum, specificity for the type amino acids near the scissile peptide bond and the position of this bond in the protein (e.g. inside or at the terminus of the amino acid string). For application in fabric washing detergents, non-specific endoproteases (which hydrolyse peptide bonds *inside* the protein) with an optimal activity at high pH are used. In this study Savinase™ [6,7], an extracellular serine endopeptidase of the subtilisin family from *Bacillus lentus*, was examined as an example of this type of enzyme. The acylenzyme mechanism by which the subtilisins catalyse the hydrolysis of peptide bonds has been studied extensively [8,9,10,11] and reviewed together with the physico-chemical properties of these enzymes [12,13,14,15]. The crystal structure of this protease has been known

[16,17] for some time and recently the structure at high resolution (0.14 nm) was published [18].

Nowadays lipases are also available for use in detergent formulations [19]. The rationale behind this use is very similar to that for proteases. Lipases can catalyse, among other reactions, the hydrolysis of ester bonds in e.g. triacylglycerol molecules, one of the main component in fats and non-mineral oils which are also an important component of stains in laundry. Of this type of enzyme, Lipolase™ [20,21], an extracellular lipase from the thermophilic fungus *Humicola Lanuginosa* S-38 [22] will also be studied here. As it has only become available much more recently than Savinase it is not yet that well known. However, the knowledge about the lipase hydrolysis mechanism in general [23,24] and the homology with the lipase from *Rhizomucor miehei* were used for the first investigation of Lipolase.

In a normal machine or hand wash of household laundry the detergent (powder or liquid) is added through a dispensing system to the wash suds. The first step is the dissolution of the enzymes and the other components of the detergent formulation such as the anionic and nonionic surfactants, salts, bleach, bleach precursors and calcium sequestrants [25]. A normal laundry consists both of soiled and clean cloth of various materials such as cotton, polyester, nylon and mixed fibres. When focusing on the mechanism of enzyme action in the wash it is clear that after dissolution, the enzyme is in solution while the soil is immobilized on the cloth. The logical next step is the adsorption of the enzyme on the stained cloth-water interface. Once adsorbed, it can catalyse the hydrolysis of one of the stain components and possibly diffuse over the interface or diffuse, maybe together with a hydrolysed soil fragment, away from the interface again. The hydrolysis of proteins and fats in stains enables dissolution of these components as the hydrolysed soil molecules are much smaller and better water soluble than the unhydrolysed stain components. Therewith other coloured organic molecules and dust particles that are fixed in a matrix of fat and/or protein are also released. Although basically this mechanism is clear establishing the importance of the various steps and alternative routes is a challenge because of the compounding complexity of the practical system and the mutual interference of the various steps in the process.

This study focuses on the examination of one step in the mechanism only: the adsorption of the enzyme from the solution on the cloth. Studying this step in a practical washing is obstructed by the following facts. The solution consists of several detergent components in relatively high concentrations while the enzyme is quantitatively speaking a minor component. Most of these abundantly present components can in principle both compete with the enzyme for the surface and interact with it in solution. The cloth is highly porous

and neither the natural fibres nor the stains are very reproducible. In addition, both the surface and the solution alter with time as hydrolysis of the soil results in removal of the stain components from the cloth into the solution. As a first step the study, reported here, was restricted to the investigation of the adsorption of enzymes from "clean" buffered solutions in absence of surfactants.

The investigation of enzyme adsorption on cloth from a "clean" solution was divided into several parts. The first part was the analysis of the techniques necessary to measure the enzyme adsorption accurately and reliably. Although several techniques are known to determine protein concentrations [26] the measurement of the proteolytic enzyme Savinase at very low concentrations proved to be a serious problem. A proteolytic enzyme hydrolyses proteins and as the enzyme is itself a protein, it is capable of self-destruction, so-called autolysis. In Chapter 2 the investigation of the adsorption measurement techniques for Savinase is reported together with the methods used to inhibit the enzyme with a small organic inhibitor PMSF (phenylmethanesulfonyl fluoride) to prevent autolysis during the adsorption experiments.

The main part of the study is the determination of the adsorption characteristics of active and inhibited Savinase and Lipolase on surfaces that are well characterised, non-porous and non-hydrolysable. The enzyme adsorption at these solid-liquid interfaces is approached in a similar way as protein adsorption is generally studied. In Chapter 3 the adsorption of Savinase on glass and on several polystyrene latices is reported under various conditions of pH, ionic strength and temperature. In Chapter 5 similar results are reported for Lipolase. The influence of the properties of Savinase on both the adsorption on these surfaces and the interaction with chromatographic column materials are reported in Chapter 4. The adsorption of six protein-engineered variants of Savinase, differing in electrostatic properties such as isoelectric point and number of charged amino acids from the naturally occurring Savinase is studied. Using the examined experimental techniques and the insight into the driving forces for Savinase adsorption previously obtained, in Chapter 6 a first step to study the adsorption of Savinase at practical solid-liquid interfaces such as polyester and clean and protein-soiled cotton is reported.

In this introductory chapter the main physico-chemical characteristics of Savinase and Lipolase are summarized. In section 1.3 an overview of the literature on protein adsorption is given together with a qualitative summary of the various interactions that are thought to determine protein adsorption. This chapter is concluded with a discussion on the interpretation of the experimentally determined adsorption values.

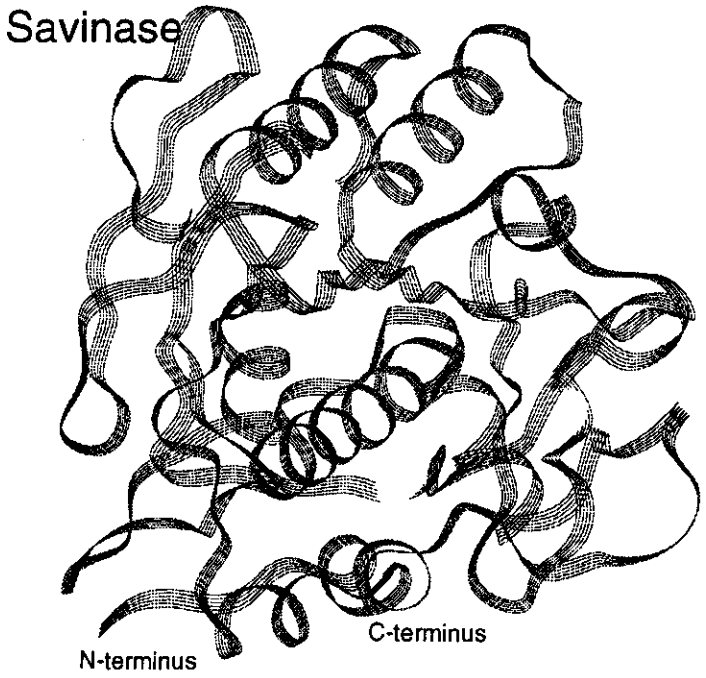

Figure 1.1 Schematic representation of the secondary and tertiary structure of Savinase as determined from the X-ray structure.

```
 1               5                  10
ala-gln-ser-val-pro-trp-gly-ile-ser-arg-val-gln-ala-pro-ala 15
ala-his-asn-arg-glu-leu-thr-gly-ser-gly-val-lys-val-ala-val
leu-asp-thr-gly-ile-ser-thr-his-pro-asp-leu-asn-ile-arg-gly
gly-ala-ser-phe-val-pro-gly-glu-pro-ser-thr-gln-asp-gly-asn
gly-his-gly-thr-his-val-ala-gly-thr-ile-ala-ala-leu-asn-asn 75
ser-ile-gly-val-leu-gly-val-ala-pro-ser-ala-glu-leu-tyr-ala
val-lys-val-leu-gly-ala-ser-gly-ser-gly-ser-val-ser-ser-ile
ala-gln-gly-leu-glu-trp-ala-gly-asn-asn-gly-met-his-val-ala
asn-leu-ser-leu-gly-ser-pro-ser-pro-ser-ala-thr-leu-glu-gln
ala-val-asn-ser-ala-thr-ser-arg-gly-val-leu-val-val-ala-ala 150
ser-gly-asn-ser-gly-ala-gly-ser-ile-ser-tyr-pro-ala-arg-tyr
ala-asn-ala-met-ala-val-gly-ala-thr-asp-gln-asn-asn-asn-arg
ala-ser-phe-ser-gln-tyr-gly-ala-gly-leu-asp-ile-val-ala-pro
gly-val-asn-val-gln-ser-thr-tyr-pro-gly-ser-thr-tyr-ala-ser
leu-asn-gly-thr-ser-met-ala-thr-pro-his-val-ala-gly-ala-ala 225
ala-leu-val-lys-gln-lys-asn-pro-ser-trp-ser-asn-val-gln-ile
arg-asn-his-leu-lys-asn-thr-ala-thr-ser-leu-gly-ser-thr-asn
leu-tyr-gly-ser-gly-leu-val-asn-ala-glu-ala-ala-thr-arg
```

Figure 1.2 Primary structure of Savinase™.

1.2 Savinase™ and Lipolase™

1.2.1 The enzymes

Savinase™ (Enzyme Classification number 3.4.21.14) is a well known [18] enzyme secreted by the alkalophilic bacterium *Bacillus lentus*. The enzyme has a high number of salt bridges that are likely to contribute to the high thermal stability. Two calcium ions can be sequestered by the enzyme in one strong and one weak binding site. Lipolase™ is less well known compared to Savinase. It is an extracellular lipase from the thermophilic fungus *Humicola Lanuginosa* S-38. The X-ray structure is not yet available and the only structural information is obtained from a comparison with the homologous lipase from *Rhizomucor miehei*.

The primary structures of Savinase and Lipolase are given in Figures 1.2 and 1.4 while the Figures 1.1 and 1.3 show schematic representations of the secondary and tertiary structures. The amino acid compositions are summarized in Table 1.1 together with the dissociation constants of the side chains of the charged amino acids [27]. The main physico-chemical characteristics are collected in Table 1.2.

1.2.2 Molecular charge of Savinase as a function of pH

The charge on a protein molecule is usually determined by titrating a solution of known protein concentration with acid or alkaline solution [28,29]. This method was used for Lipolase as is reported in Chapter 5. However, for Savinase this method can not be used as the enzyme is highly active in the pH range of interest (pH=7 to 11). Autolysis of the enzyme is considerable at the high Savinase concentrations necessary for an accurate titration experiment. Every hydrolysed peptide bond generates a new C-terminus (-COOH) group and a N-terminus ($-NH_2$) group. As the carboxylic acid dissociates above pH=4 and the amino group takes this proton up only at pH values below their pK (pH=8 to 9), the hydrolysis of a peptide bond at high pH corresponds to the release of one proton. The titration curve of active protease will therefore not represent the net charge on the enzyme. Using inhibited Savinase does not solve this problem as autolysis was only diminished but not fully prevented (see Chapter 2). Therefore the molecular charge on the Savinase molecule was calculated instead of determined experimentally. The calculation is based on equation (1) published by Tanford and Kirkwood [30] that uses Debye-Hückel theory to determine the Gibbs energy of charging an impenetrable sphere. In this calculation the pK values of the charged amino acids are corrected for the total charge of the protein molecule assuming this charge to be evenly spread over the spherical molecule.

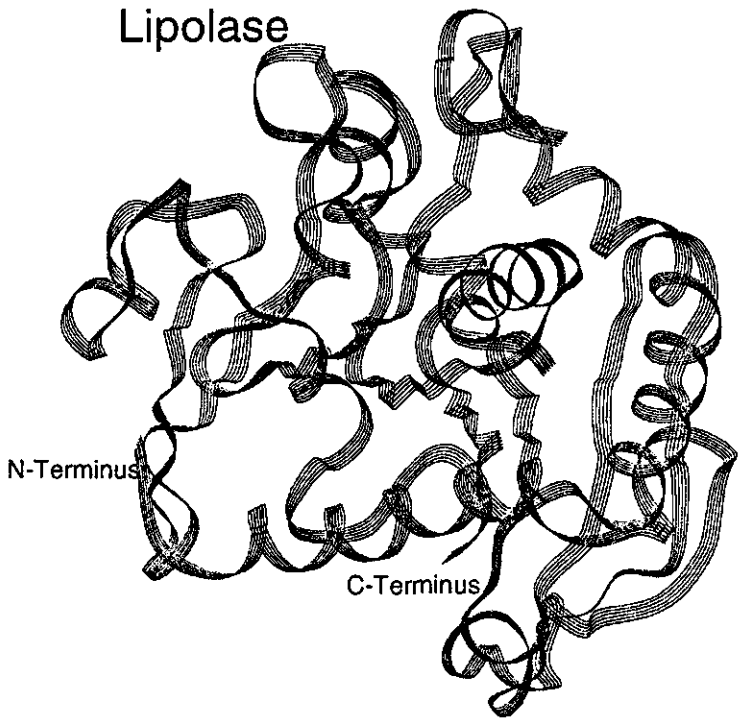

Figure 1.3 Schematic representation of the secondary and tertiary structure of Lipolase as determined from the X-ray structure of the lipase from *Rhizomucor miehei*.

```
 1               5                  10
glu-val-ser-gln-asp-leu-phe-asn-gln-phe-asn-leu-phe-ala-gln  15
tyr-ser-ala-ala-ala-tyr-cys-gly-lys-asn-asn-asp-ala-pro-ala
gly-thr-asn-ile-thr-cys-thr-gly-asn-ala-cys-pro-glu-val-glu
lys-ala-asp-ala-thr-phe-leu-tyr-ser-phe-glu-asp-ser-gly-val
gly-asp-val-thr-gly-phe-leu-ala-leu-asp-asn-thr-asn-lys-leu  75
ile-val-leu-ser-phe-arg-gly-ser-arg-ser-ile-glu-glu-thr-gly
ser-gly-asn-leu-asn-phe-asp-leu-lys-glu-ile-asn-asp-ile-cys
ser-gly-cys-arg-gly-his-asp-gly-phe-thr-ser-ser-trp-arg-ser
val-ala-asp-thr-leu-arg-gln-lys-val-glu-asp-ala-val-arg-glu
his-pro-asp-tyr-arg-val-val-phe-thr-gly-his-ser-leu-gly-gly 150
ala-leu-ala-thr-val-ala-gly-ala-asp-leu-arg-gly-asn-gly-tyr
asp-ile-asp-val-phe-ser-tyr-gly-ala-pro-arg-val-gly-asn-arg
ala-phe-ala-glu-phe-leu-thr-val-gln-thr-gly-gly-thr-leu-tyr
arg-ile-thr-his-thr-asn-asp-ile-val-pro-arg-leu-pro-pro-arg
glu-phe-gly-tyr-ser-his-ser-ser-pro-glu-tyr-trp-ile-lys-ser 225
gly-thr-leu-val-pro-val-thr-arg-asn-asp-ile-val-lys-ile-glu
gly-ile-asp-ala-thr-gly-gly-asn-asn-gln-pro-asn-ile-pro-asp
ile-pro-ala-his-leu-trp-tyr-phe-gly-leu-ile-gly-thr-cys-leu
```

Figure 1.4 Primary structure of Lipolase™.

Table 1.1 Composition of Savinase and Lipolase with the pK values of the charged residues.

Amino acid	pK value	Savinase	Lipolase	Amino acid	pK value	Savinase	Lipolase
Ala		40	21	Leu		19	20
Arg	12.5^{+}	8	14	Lys	10.5^{+}	5	7
Asn		22	18	Met		3	-
Asp	3.7^{-}	5	19	Phe		2	15
Cys	8.3^{*}	-	6	Pro		13	12
Gln		10	6	Ser		34	18
Glu	4.3^{-}	5	13	Thr		17	20
Gly		35	29	Trp		3	3
His	6.0^{0}	7	6	Tyr	10.1^{0}	7	10
Ile		9	15	Val		25	18

$^{+}$, $^{-}$ indicates the sign of the charge of the amino acid at pH=8.
^{0} indicates that the amino acid is uncharged at pH=8.
* as the cysteines are all involved in sulphide bridges they are assumed not to be titratable.

Table 1.2 Physico-chemical characterisation of (PMS-)Savinase and Lipolase.

Properties	(PMS-)Savinase	Lipolase
Molecular weight	27,000 g mol^{-1}	27,000 g mol^{-1}
Isoelectric point	pH = 10	pH = 4.3
Shape	sphere	ellipsoid
Dimensions	radius of 2.3 nm	5.3 x 5.0 x 4.1 nm^3
Secondary structure from CD spectroscopy*	α-helix: 27 $\pm$ 2% β-sheet: 49 $\pm$ 2%	α-helix: 36 $\pm$ 3% β-sheet: 28 $\pm$ 8%
Residual activity of PMS-Savinase compared to active Savinase	0.1%	

* Secondary structure is determined using the program "CONTIN" [31,32].

$$pH = pK_{int,i} + \log(\alpha_i/(1-\alpha_i)) - 692 \cdot Z \cdot w \qquad (1)$$

where $pK_{int,i}$ is the intrinsic dissociation constant of amino acids of class i, α_i is the dissociated fraction of class i, Z is the number of elementary charges on the molecule and w is an electrostatic interaction factor, which for a spherical molecule with a symmetrical charge distribution is given by:

$$w = e^2 (1 - \kappa a/(1+\kappa d)) / 2\epsilon akT \qquad (2)$$

where e is the elementary charge, ϵ is the dielectric permittivity of the solvent, a is the radius of the protein molecule, κ is the Debye-Hückel parameter, d is the distance of closest approach between the centres of an average ion and the protein, k is Boltzmann's constant and T is the absolute temperature (all constants in SI units). The number of elementary charges on the molecule is not only defined by the dissociation of the amino acids but also by the number of ions bound to the protein (in the case of Savinase e.g. Ca^{2+}-ions) as shown in equation (3):

$$Z = Z_{ion} + Z_{H,max} - \Sigma_i n_i \cdot \alpha_i \qquad (3)$$

where Z is the total charge, Z_{ion} is the charge caused by bound ions, $Z_{H,max}$ is the charge obtainable upon full protonation of the amino acids, at very low pH ($pH < 1$), n_i is the number of amino acids of class i and α_i is the dissociated fraction of class i. The titration curve can be calculated by simultaneous solution of equation (1) for all classes of amino acids present in the molecule [33].

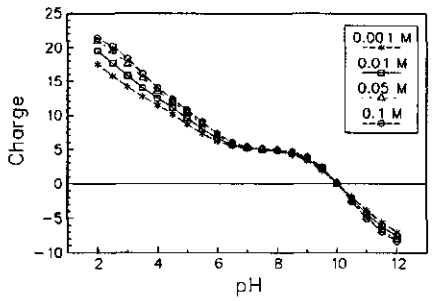

Figure 1.5a Calculated titration curves of Savinase at various ionic strengths (symbols do not represent measuring points).

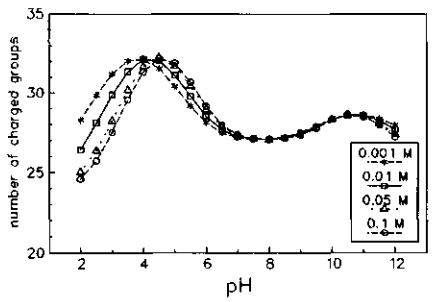

Figure 1.5b Calculated number of charged groups on Savinase as function of pH at various ionic strengths (symbols do not represent measuring points).

The titration curves of Savinase (Figure 1.5a) were calculated at various ionic strengths assuming one calcium ion to be bound per enzyme molecule. In Figure 1.5b the total

number of charged residues is shown as a function of pH at various ionic strengths. All positively and negatively charged amino acids with an individual charge larger than 0.1 elementary unit are counted as charged. It can be concluded that, although the net charge varied, the number of charged residues was virtually constant between pH=8 and pH=11.

1.3 Protein adsorption

Enzymes are proteins and it is a long time ago since that was doubted [34]. The adsorption of enzymes on non-hydrolysable solid-liquid interfaces is therefore expected to be similar to protein adsorption in general. Protein adsorption has been widely investigated both because of its practical and academic interest. The adsorption of proteins from body fluids such as blood [35,36,37], plasma [38,39], urine [40,41], saliva [42] and tears [43,44] onto implanted materials, teeth and contact lenses can cause blood coagulation and biofouling by subsequent adhesion of cells and bacteria [45,46]. In these cases protein adsorption is studied with the aim to determine the conditions and sorbent properties where biofouling is mimimalized [47]. In other cases the adsorption of one or more proteins is a desired feature, for example for biosensors [48], the immobilisation of biocatalysts [49] and the stabilisation of emulsions [50,51]. Good overviews of the complete field of protein adsorption research can be obtained from the tutorial review by Andrade and Hlady [52] and the proceedings of the 192nd meeting of the American Chemical Society in 1986 [53].

Most natural protein solutions are mixtures and also contain a variety of non-proteinaceous components. In order to study the principles of protein adsorption on solid-liquid interfaces, the complex biological systems had to be simplified. Depending on the field of research and the intended application, experimentators have chosen various simplified systems that modelled specific properties of the natural system. This variety in experimental systems and conditions hampered a systematic comparison and interpretation of the results in terms of the adsorption mechanism. It is therefore not surprising that in the past 10 years over 100 review articles were published on protein adsorption. In this section we will refrain from reviewing all of this but restrict ourselves to give a summary of the mechanisms of protein adsorption in general.

Protein adsorption is such a complicated process because it is determined by a delicate balance between several attractive and repulsive interactions. The relative importance of the various "driving forces" is different for various classes of proteins. Therefore it is difficult to distinguish the common driving forces behind their adsorption and to predict the behaviour of a particular protein at any solid-liquid interface. Recent reviews

[54,55,56,57,58,59] showed that the interactions that determine the adsorption of proteins can be categorised as follows.

1. *Protein-surface interactions.* These interactions can have various origins such as electrostatic attraction or repulsion between the protein and the interface and/or Van der Waals attraction. The most important contribution to the long range electrostatic interaction is the Coulomb interaction due to the *net* charge of the surface and the protein. The net charge on the protein results from the dissociation of various amino acids located mostly on the periphery of the molecule (Table 1.1). Any non-homogeneity in the charge distribution, leading to a dipole moment and so-called "mosaic charge distribution", can also contribute to the electrostatic interaction (see also Chapter 4). The electrostatic interaction is further complicated by the incorporation of ions into the layer between the interface and the protein [60,61,62].
2. *Dehydration of interfaces* on the outside of the protein and on the solid surface. Dehydration of hydrophobic interfaces promotes protein adsorption while the dehydration of hydrophilic ones opposes it. As protein molecules are large (radii of 2 to 4 nm) compared to a water molecule (radius of 0.14 nm) adsorption results in the release of a large number of water molecules. Therefore this interaction is very important and often overrules any of the other "driving forces".
3. *Structural changes in the protein molecule upon adsorption.* The densely folded structure of globular proteins in solution is mainly attributed to the fact that hydrophobic interactions in the interior of the molecule (i.e. prevention of hydration of these residues) are stronger than the intramolecular electrostatic repulsion in the perifery of the molecule and the loss of entropy upon folding. When the protein is brought in contact with an interface the balance between these forces may change because the hydrophobic interior of the molecule can prevent hydration in an alternative way by positioning itself against the interface. This unfolding will be promoted by stronger electrostatic repulsion between the charges on the adsorbed protein. Unfolding of the adsorbed protein will increase the surface area occupied by the protein. It will also result in a lower electrostatic repulsion between like charges in the adsorbed protein and an increased entropy of the protein. The extent of unfolding of the protein also depends on the characteristics of the sorbent [63]. If the interface is hydrophobic, unfolding will be enhanced. Also, charges on the interface can compensate those on the adsorbed protein, therewith changing the intramolecular electrostatic repulsion and altering the degree of unfolding.
4. *Surface coverage dependent lateral interactions*, caused by the accumulation of protein molecules at the interface. The electrostatic repulsion between the equally

charged molecules and the resulting loss of entropy of the system oppose completion of monolayer coverage. Dipole-dipole interaction between the proteins on the surface can be either repulsive or attractive depending on the degree of alignment of the molecules.

Quantitative determination of the change in Gibbs energy upon adsorption [56] is obstructed by the fact that the mechanism for folding and unfolding of a protein is not yet known. Therefore it is not possible to predict the conformation in which the protein will adsorb. Not only the quantification of interaction 3 but also the determination of the size of the dehydrated surface area (interaction 2) is obstructed by this as both factors depend strongly on the degree of unfolding.

Although all four categories of interactions play a role in the adsorption of every protein the relative importance differs. In general, two classes of proteins are distinguished namely "soft" and "hard" proteins. The difference is based on the importance of structural alterations upon adsorption (3). "Soft" proteins (such as Human Plasma Albumin [64,65,66] and Immunoglobulin G [67]) are thought to unfold partially upon adsorption. This degree of unfolding increases with increasing net charge on the protein-interface complex because of repulsive electrostatic *intra*molecular repulsion. The gain in Gibbs energy upon unfolding may contribute to a significant adsorption on hydrophilic surfaces, even under repulsive electrostatic conditions (HPA on AgI at high pH [54]).

Structural alterations are not that important for the so-called "hard" proteins (such as RNase [64] and Myoglobin [68]). Dehydration of hydrophobic interfaces (2) and electrostatic interactions (1) are the main driving forces for adsorption of this category of proteins. These proteins adsorb on all hydrophobic interfaces independently of the charge of the surface. And it is typical for the "hard" proteins to adsorb on hydrophilic surfaces only under conditions of attractive electrostatic interactions (negligible adsorption of RNase on hematite at low pH [69]).

1.4 The adsorption isotherm: experimental tool for investigation of the adsorption mechanism

In order to determine which of the above-mentioned interactions play an important role in enzyme adsorption, the adsorption isotherms have to be measured under several experimental conditions. The information that can be gained from an adsorption isotherm is discussed in this section. In Figure 1.6 the relations between the various kinds of adsorption experiments are summarized. After the protein solution is brought in contact with the surface, it takes a certain time before the adsorption and the protein

concentration in solution reach their equilibrium values (Figure 1.6a). The kinetics of protein adsorption is an important subject of many studies especially in relation to the competition between proteins [70,71,72,73,74]. In this study the kinetics of enzyme adsorption will not be investigated in detail.

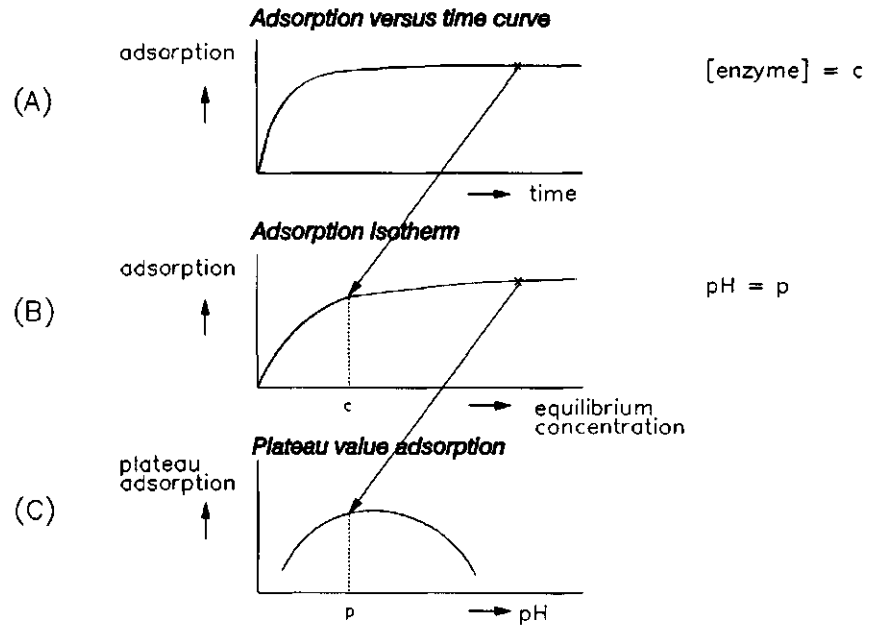

Figure 1.6 Schematic overview of relations between various adsorption experiments.

An adsorption isotherm is obtained when the equilibrium adsorption values are determined as a function of the equilibrium protein concentration in solution. The isotherm gives a relation between the amount of protein per surface area and the concentration of the protein in solution when adsorbate and solution are in equilibrium (Figure 1.6b). The adsorption isotherm of a monomeric protein is independent of the ratio between the surface area and the volume of the protein solution and depends only on experimental conditions such as pH, ionic strength and surface charge (see Chapter 2). In an adsorption isotherm two parts can be distinguished. The rising initial part at low concentrations indicates the affinity of the protein for the surface. When the isotherm rises very steeply the isotherm is called a "high affinity" isotherm. This means that almost all protein that is present in the system will be at the surface and the amount remaining in solution is minimal. The slope of the isotherm at low surface coverage is a measure of the first three of the four interactions discussed in the previous section.

In general the adsorption increases up to an amount where the total surface is saturated with protein. This amount will be called the "plateau value" of the isotherm (or Γ_{plat}) and in general this amount depends on the experimental conditions. The plateau value not only reflects the affinity of the protein for the surface but also the repulsive surface coverage-dependent lateral interactions (4) and the conformation of the adsorbed protein. The shape of a typical protein adsorption isotherm is very similar to the well known Langmuir gas adsorption isotherm [75] and in some publications the protein adsorption is interpreted accordingly [76,42]. However, the typical irreversibility of protein adsorption [54,77,78] against dilution (so-called hysteresis of the isotherm) shows that one of the essential conditions for a Langmuir isotherm is not fulfilled in case of the adsorption of most proteins.

The dependence of this plateau value can be expressed as a function of the experimental conditions such as pH or ionic strength at which the isotherms were measured (Figure 1.6c). The latter type of representation (Figure 1.7) clearly shows the differences between the two categories of proteins indicated above. At pH value higher and lower than the isoelectric point of the protein-sorbent complex the "soft" proteins unfold more strongly than at the isoelectric point. In the former situation the surface area occupied per adsorbed protein molecule is larger thus reducing the maximum amount of protein adsorbed. At any pH value the complete interface is covered with (unfolded) protein. The plateau value adsorption of a "hard" protein on a hydrophobic interface is virtually pH independent as the protein does not unfold upon adsorption. Investigation of the pH dependence of the plateau value of the adsorption isotherms can therefore indicate whether unfolding is an important driving force for adsorption. Analysis of the slope and the plateau value of the adsorption isotherm at several surfaces under various conditions regarding pH, ionic strength and temperature can then be used to determine which of the other interactions are important for the adsorption of Savinase and Lipolase.

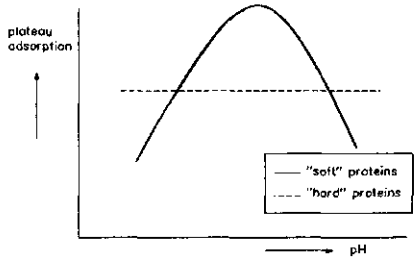

Figure 1.7 *Schematic representation of the variation of plateau value with pH on a hydrophobic interface for hard and soft proteins.*

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CHAPTER 2

EXPERIMENTAL TECHNIQUES FOR SAVINASE ADSORPTION MEASUREMENT

Abstract

In view of the prevailing experimental conditions that will be encountered in later chapters, a sensitive determination of Savinase concentrations is mandatory. In this chapter four methods will be discussed, namely the Radio-active Tracer Technique (RTT), UV 280 nm extinction measurement, Lowry colouring method and a method based on the detection of the proteolytic activity of Savinase on AAPF (N-succinyl-L-Alanyl-L-Alanyl-L-Prolyl-L-Phenylalanine-p-Nitroanilide).

The results showed that the Radio-active Tracer Technique method could not be used for the measurement of Savinase adsorption because the unlabelled enzyme adsorbed preferentially over the labelled one. The latter was shown to be degraded by auto-digestion (autolysis) during the labelling reaction and subsequent storage. The competition between the numerous protein fragments resulted in a surface area- and volume-dependent adsorption comparable to the adsorption from polydisperse polymer solutions.

The detection limit of the UV 280 nm extinction method was too low to detect the rising part of the adsorption isotherm. Small polystyrene latex particles remained in solution even after centrifugation and light scattering from these particles obstructed accurate determination of the concentration after contact with the polystyrene surface. The Lowry colouring method gave reproducible and reliable results but can not be used to detect Savinase in a protein mixture. For that purpose the so-called "AAPF" method will be used to measure proteolytically active Savinase. As autolysis becomes more rapid at higher enzyme concentrations this method is restricted to the concentration regime of 0.1 $\mu g\ ml^{-1}$ to 30 $\mu g\ ml^{-1}$.

2.1 Introduction

In principle there are two different ways of determining adsorbed amounts. The first is to measure directly the amount of protein that is on the surface after a certain contact time. However, this "actual" adsorbed amount can only be determined if it is possible to

detect the protein in the adsorbed state or if it can be removed quantitatively. This direct way is experimentally more difficult to execute than the second method: the so-called "depletion measurement". In this method the protein adsorption is calculated from the amount of protein that disappears from solution after bringing it into contact with a known surface area. This method requires a relatively large surface area of the sorbent, so that adsorption results in a significant decrease of protein concentration in solution. The latter method was used in this study. The adsorption measurement by depletion requires of the determination of four parameters: the volume of the protein solution, the surface area in contact with it and the protein concentration before and after a certain contact time.

The key part of an adsorption experiment is the determination of the protein concentration before and after contact with the surface. In the literature many different methods are used to detect proteins in solution [1]. These methods can roughly be divided into three groups. The most elegant one is based on detection of intrinsic properties of the protein without addition of any reactant either before the adsorption experiment or before the concentration detection; an example is the UV 280 nm extinction. The second group consists of methods that are based on the detection of a product of a reaction between the protein and components which are added to the enzyme solutions *after* the adsorption is completed by separation of surface and solution; examples are the Lowry and the BCA (bicinchoninic acid) colouring method and enzymatic activity measurement on model substrates such as AAPF (N-succinyl-L-Alanyl-L-Alanyl-L-Prolyl-L-Phenylalanine-p-Nitroanilide). The third group of methods is based on an essentially different principle. A small amount of labelled protein (with a very low detection limit) is added to the bulk of the protein *before* the adsorption experiment. The C_0 and C_e are then calculated from the experimental detection of the labelled protein only. Examples from this group are the radio-active and fluorescent tracer techniques.

In this study we examined four methods of protein concentration detection, each belonging to one of the above mentioned groups namely the radio-active tracer technique, the Lowry colouring method, UV 280 nm extinction detection and detection of enzymatic activity on AAPF. In section 2.3 these methods will be described in detail.

Determination of the adsorbed amount of a proteolytic enzyme such as Savinase is complicated by the fact that the enzyme can hydrolyse itself, so-called "autolysis", during the experiment. In this adsorption study we therefore used Savinase that was inhibited by a small, uncharged molecule, PMSF (phenylmethanesulfonyl fluoride). PMSF was bonded covalently to the Serine residue in the active site of the enzyme therewith reducing the enzymatic activity to about 0.1% of the uninhibited Savinase.

2.2 Materials

2.2.1 Savinase

Savinase was isolated from a commercial preparation (ex NOVO-Nordisk, Denmark) by the Bio- and Nutrition Sciences Department, URL Vlaardingen. To this end 100 gram of the preparation was dissolved in 800 ml 0.01 M NaAc, pH=5.0, and centrifuged. The solution was dialysed against water using a hollow fibre dialyser (Nephross Andante HF-Organon Teknika) until the conductivity was smaller than 1.0 mS. After dialysis approximately 250 ml CM-Trisacryl (ex LKB-Pharmacia) ion exchange resin, equilibrated with acetate buffer, was added and the mixture was stirred (Heidolff) overnight at 5°C. After washing the ion exchange resin was washed with acetate buffer, it was transferred to a column (K50, 15 cm). The Savinase was eluted by applying a NaCl gradient from 0 to 0.5 M in the same buffer. The fraction containing Savinase (approximately 300 ml) was decoloured by batchwise addition of 100 ml DEAE-Sepharose FF (ex LKB-Pharmacia). After 1 hour incubation the gel was removed and the fraction was concentrated by ultrafiltration over an Amicon YM10 filter to 100 ml. The purified Savinase (batch 90.1) was stored at −20°C in a stabilizing buffer solution (final concentrations 33% glycerol in 7 mM acetate at pH=5) at a concentration of about 83 mg ml^{-1}.

Prior to use the Savinase is separated from the glycerol/acetate-buffer solution by gel filtration, using a PD-10 column (Sephadex G-25M no. 17-0851-01, ex Pharmacia). The separation procedure is described in Appendix 2.1.

2.2.2 PMSF-inhibited Savinase

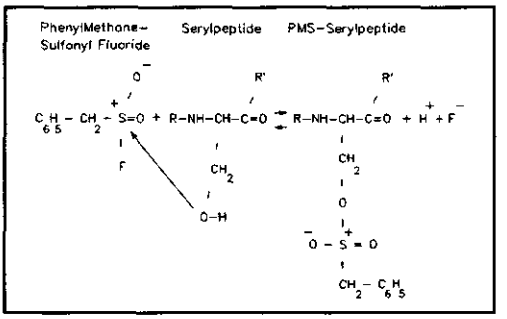

Figure 2.1 Schematic representation of the inhibition reaction.

Principle

In order to prevent autolysis (hydrolysis of the enzyme by itself) during the adsorption experiment we inhibited the enzyme. In the literature the inhibition of proteases has been examined extensively [2,3,4,5,6]. As we are interested in the properties of active Savinase the inhibitor or inhibition reaction should not be allowed to influence the adsorption characteristics of the enzyme. This requirement excludes *reversible* inhibitors such as Bacitracin [7,8] as this type of inhibitor is to be added to the solution during the adsorption experiment itself. It could

then not only inhibit the enzyme but also compete with the enzyme for the surface or change the solubility of the enzyme. We looked for an uncharged, small, inhibitor that binds irreversibly to a serine protease at the time scale and under the experimental conditions used in this study.

In the literature [9,10,11,12] a well known inhibitor of serine proteases is the noncompetitive inhibitor PMSF (phenylmethanesulfonyl fluoride). Under neutral pH conditions the small uncharged PMSF molecule reacts with the serine active site of the protease by a nucleophilic substitution reaction and after binding it blocks the active site. A schematic representation of the reaction mechanism is shown in Figure 2.1.

The inhibition reaction is in principle reversible at high pH (pH > 8.5) [13]. A nucleophilic substitution of PMSF by OH^- gives phenylmethanesulfonate and the original serylpeptide, thereby restoring the proteolytic activity of the enzyme. The reactivation reaction is controlled by the pH and it is slow compared with the time scale of our adsorption experiments (contact time about 1 hour).

Inhibition procedure

The inhibition of Savinase can cause autolysis during two periods. The first period is during the inhibition reaction itself and the second period is during dialysis and storage when the residual activity can cause degradation of mainly inhibited enzyme. To prevent a loss of enzyme and contamination of the sample by autolysis fragments it is necessary to accomplish complete inhibition at the highest possible rate.

The inhibition procedure published for Chymotrypsin [12] was therefore modified with an inhibitor/enzyme (I/E) ratio of 1000 in order to reach a more complete inhibition in a shorter time period so that less autolysis could occur. As the solubility of PMSF in water at room temperature is of the order of 1 mM the enzyme concentration during the inhibition reaction was no more than 1 μM. The residual activity was about 0.1% after 10 minutes with an I/E ratio of 1000. The Reversed Phase HPLC purity check (2.4.1) showed that the solution contained hardly any Savinase fragments. The preparation procedure is summarized in Appendix 2.1. In the following Savinase inhibited with PMSF will be indicated by PMS-Savinase.

2.2.3 Polystyrene latices

The emulsifier-free latex was kindly supplied by Dr. W. Norde (Agricultural University Wageningen). It was prepared according to the procedures published by Furusawa et al [14]. The negative surface charge is attributed to sulphate groups and the surface

charge was determined by potentiometric titration to be $-11.6\ \mu C cm^{-2}$. The surface area was calculated from the specific surface diameter [15], (the $D_{3,2}$ or Sauter diameter equal to 495 nm) that was determined with a SympaTec Helos particle sizer. An Electron Microscope picture (Figure 2.2) of latex A shows that the latex particles are spherical but not perfectly monodisperse.

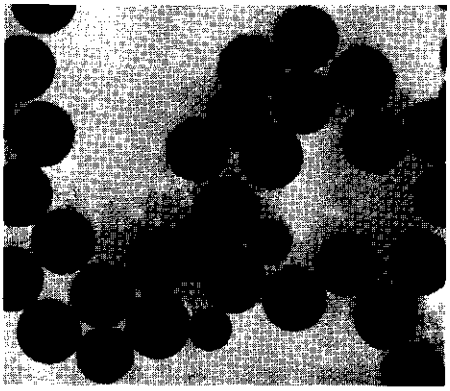

Figure 2.2 Electron Microscope picture of latex A.

2.2.4 Buffer solutions

All adsorption experiments were performed in buffer solutions. In order to prevent differences in specific interactions with either the enzyme or the latex surface we used the same buffer system for all solutions with pH-values ranging from 7 to 11.

This system was sodium tetraborate, $Na_2B_4O_7.10H_2O$, normally known as Borax (ex Merck) that was brought to the desired pH and ionic strength by HCl/NaOH and NaCl respectively. A concentrated buffer solution was added to the enzyme solution to obtain final concentrations of 0.0028 M Borax, 0.0044 M HCl for pH=8 and 0.0028 M Borax, 0.0040 M NaOH for pH=10 conditions. The ionic strength was calculated assuming that the borate-ion is divalent. However, the absolute value of the ionic strength is unknown because the borate ions can form large complexes [16].

2.3 Experimental methods

2.3.1 Adsorption measurement

The adsorption experiments were performed by addition of 1 ml enzyme solution to 0.2 ml PS-latex in an Eppendorf 2 ml centrifuge tube. During the contact time the tubes were shaken horizontally in a thermostatted water bath. The incubation was stopped by

separation of enzyme solution and surface by centrifugation in an Eppendorf 5415C centrifuge for 10 minutes at 16,000 g (14,000 rpm). As the density of polystyrene only marginally exceeds that of water ($\rho_{PS}=1.05\ 10^3$ kg m^{-3}) centrifugation did not lead to a complete separation of all PS-particles from the solution. Therefore the supernatant was pipetted very carefully from the upper part of the tube only.

Unless mentioned otherwise all results reported in this chapter have been measured at the following experimental conditions: ionic strength=0.01 M, temperature=3°C, volume of enzyme solution=1.5 ml and surface area=0.02 m^2.

2.3.2 Concentration determination by the Radio-active tracer technique

Principle
Before the start of an adsorption experiment a small amount of PMS-[^{3}H]-Savinase was added to the bulk of non-labelled PMS-Savinase. The concentrations of the original solutions were determined by UV 280 nm extinction. The amount of radio-activity in the resulting solution was determined by liquid scintillation counting. The specific radio-activity (ratio of radio-activity and total protein concentration) was then used to calculate the starting (C_0) and equilibrium (C_e) concentrations from the experimentally determined amount of labelled protein only.

The implicit assumption underlying this method is that the labelled and non-labelled PMS-Savinase have identical adsorption properties. If this is not the case the competition between labelled and unlabelled protein for the surface is studied instead of the intrinsic adsorption characteristics of the protein itself. This phenomenon has been discussed frequently in the literature [17,18,19,20,21] for various proteins and labelling procedures and it will be treated for (PMS)-Savinase in section 2.4.1. The important advantages of this method are the low detection limit (of the order of 10 pM) and the fact that it enables one to detect one specific protein in the presence of others.

Preparation of radio-actively labelled PMS-[^{3}H]-Savinase and [^{3}H]-Savinase
The protease was radio-actively labelled by reductive methylation of the -NH_2 group of both the N-terminus and the lysines with tritium-labelled sodium cyanoborohydride. The principles of this labelling reaction were described before [22,23,24].

In this study we used two different labelling procedures. The first (indicated as "old") was based on the method published by Dottavio & Ravel [22]. However, this "old" procedure proved to be unsuitable for a proteolytic enzyme, as will be shown in section 2.4.1. The main reason was that efficient labelling required relatively high enzyme concentrations

which led to autolysis of the enzyme during the labelling reaction. Every peptide bond that was broken before or during the labelling reaction resulted in one extra -NH_2 group on the newly formed N-terminus of the fragment which was subsequently radio-actively labelled. If dialysis was not performed rigorously enough these labelled fragments contaminated the enzyme sample. Therefore the "new" labelling procedure consisted of a longer and more effective dialysis resulting in a lower concentration of fragments left in the final solution.

Another improvement concerned the labelling of inhibited enzyme only. In the "old" procedure the inhibition was preformed immediately *before* the labelling. Reversal of this order (Appendix 2.2) resulted in a reduction of the reaction time at *high* concentration and decreased the amount of autolysis. Another possibility appeared to be starting the labelling reaction with separately prepared PMS-Savinase (inhibition reaction according to Appendix 2.1). This, however, did not result in a monodisperse [^{3}H]-PMS-Savinase. An improved labelling procedure for both active and PMS-Savinase is given in Appendix 2.2. The three batches of PMS-[^{3}H]-Savinase that were prepared with the new labelling procedure will be indicated by the month in which they were prepared (e.g. 8-'90).

Experimental procedure
The radio-activity of the 0.1 to 0.2 ml samples of the protein solutions was measured using Emulsifier Scintillator 299 TM as the scintillation liquid and the TRI-CARB Analyzer (type 1900 CA or 2000). Before mixing 7 ml of the scintillation liquid with the enzyme solution, the latter was diluted with water up to 1 ml to achieve optimal solubilization of the enzyme in the emulsion during counting. All samples were counted twice for 10 minutes. The minimum counting rate had to be as high as 150 cpm (counts per minute) in order to get reliable results.

2.3.3 Concentration determination with UV 280 nm extinction detection

Principle
UV light between 240 and 300 nm is absorbed by three aromatic amino acids (tryptophan, tyrosine and phenylalanine) which are present in most proteins [25]. When the extinction coefficient of a protein is known a simple spectrophotometric determination of the extinction in this region is sufficient to determine the protein concentration.

This method detects all UV-absorbing components present in the solution and can therefore only be used for samples containing one known protein without UV-absorbing contaminants. The detection limit is of the order of 0.5 μM but it depends strongly on the number of aromatic amino acids in the examined protein.

Experimental procedure

A typical Savinase UV spectrum is shown in Figure 2.3. The peak at 280 nm is not completely separated from the one at shorter wavelength. In order to determine the extinction caused by the aromatic amino acids a three-point method is often used ($E_{3p,280nm} = E_{280nm} - 0.5(E_{250nm} + E_{310nm})$; see Figure 2.3). However, the extinction of enzyme solutions that have been in contact with PS-latex is magnified by light scattering of small PS-particles (visible in the E.M. photograph Figure 2.2) still present after centrifugation. Because the contribution of light scattering to the total extinction is higher at lower wavelengths the three-point detection could not be used here. Therefore a single point measurement was used and the concentration calculated using a calibration curve detected in the same way.

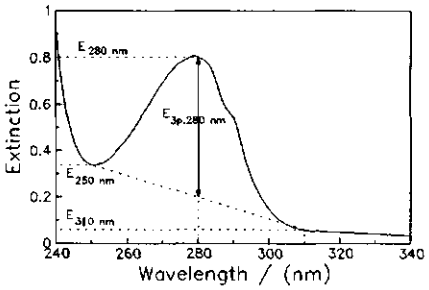

Figure 2.3 Typical UV spectrum of PMS-Savinase.

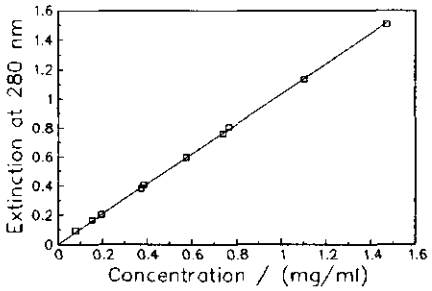

Figure 2.4 Calibration curve of Savinase UV absorption at 280 nm with optical path length of 1 cm.

The use of small Hellma Suprasil 104B-QS, 1 cm path length, quartz cuvettes enables measurement of samples as small as 0.5 ml.

Extinction coefficient of Savinase

The extinction coefficient was determined experimentally as the slope of a calibration curve of the extinction at 280 nm against the concentration in dry-weight per volume (Figure 2.4). The enzyme used to determine this calibration curve was highly purified and isolated on a PD 10 column in a way that minimizes the salt content of the isolated enzyme. In order to minimize the water content of the enzyme it was rigorously freeze-dried and stored in a desiccator. The experimentally determined extinction coefficient of Savinase is $1.03\ l\,g^{-1}\,cm^{-1}$.

The theoretical value can be calculated from the amino acid composition of Savinase and the molecular extinction coefficients of the single amino acids dissolved in water. The method of calculation is described in [26]. It has been shown there that the extinction of an amino acid in water does not differ dramatically from the extinction when it is

incorporated in a protein. The molecular extinction coefficient calculated in this way for Savinase is 26030 l mole^{-1} cm^{-1}.

In order to compare the calculated molar coefficient and the experimentally determined one it is necessary to know the molar mass. This is not trivial for proteins because two kinds of "molar masses" can be distinguished. The first one is the sum of the masses of the amino acids and the second includes also salt ions, fatty acids and other strongly bound small molecules. In our opinion the latter should be used for the comparison of coefficients because in purified freeze-dried protein samples these other components will still be present. However, Gill and Von Hippel [26] used the sum of masses of the amino acids as the molar mass. This favours the comparison between the theoretical and experimental values of the examined proteins.

Table 2.1 Comparison of theoretical and experimental extinction coefficient.

	M_w= 26,700 g mol^{-1}	M_w= 30,000 g mol^{-1}
ϵ_{th} (l g^{-1} cm^{-1})	0.97	0.87
$(\epsilon_{th}-\epsilon_{exp})/\epsilon_{exp}$	-6%	-16%

ϵ_{exp} = 1.03 l g^{-1} cm^{-1}
ϵ_{th} = 26,030 l mol^{-1} cm^{-1}

The theoretical and experimental coefficients of Savinase are compared in Table 2.1 using two molar masses. This shows that the experimentally determined coefficient compared well with the theoretical one when the same comparison was made as reported by Gill and Von Hippel. However, it is clear that the use of a higher molar mass results in a less favourable comparison between the two methods. In this report the experimentally determined coefficient of 1.03 l g^{-1} cm^{-1} and a molar mass of 27,000 g mol^{-1} will be used. In order to avoid errors due to the uncertainty in the value for the molar mass both the concentration and the adsorbed amount will be given in weight instead of molar units. The molar mass will only be used for calculation of the surface coverage.

2.3.4 Concentration determination with the Lowry colouring method

Principle

The Lowry protein detection method [27,28] is based on a reaction between the peptide bonds of the protein and added copper sulphate at pH=13. After reduction of Cu^{2+} to Cu^{+}, the latter ion associates with Folin reagent giving an intense blue-coloured complex. The extinction of this complex is detected spectrophotometrically at 750 nm. The protein concentration can be calculated by comparison with a previously made (non-linear) calibration curve. The detection limit is in the order of 0.1 μM for Savinase.

Special care must be taken when components other than the protein (e.g. some surfactants) are present in the solution because they could influence the colouring reaction [29]. The method is less vulnerable to disturbances by small particles (dust and/or PS-particles) than e.g. UV 280 nm detection because the extinction is measured at a relatively high wavelength.

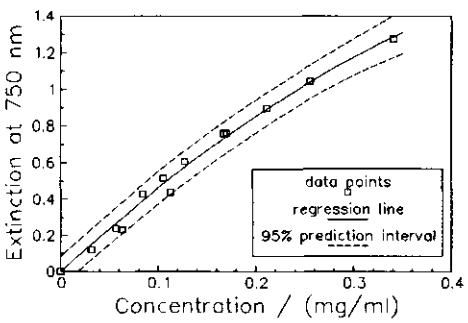

Figure 2.5 Lowry colouring calibration curve of Savinase with 95% prediction interval (optical path is 1 cm).

Experimental procedure

The procedure for the colouring reaction is based on the one reported by Reintjes [30]. Both the procedure and the chemicals are given in Appendix 2.3. The calibration curve of Savinase (Figure 2.5) is based on concentrations determined with UV 280 nm extinction. The data points can be fitted adequately with the following equation:

$$E_{750nm} = -3.5092\ [Sav]^2 + 4.9720\ [Sav]\ ;\ \text{with}\ 0 < [Sav] < 0.34\ \text{mg ml}^{-1}.$$

2.3.5 Concentration determination by measurement of proteolytic activity on AAPF

Principle

A method to determine the concentration of a protease is detection of its proteolytic activity on a soluble substrate. The tetrapeptide N-succinyl-L-Alanyl-L-Alanyl-L-Prolyl-L-Phenylalanine-p-Nitroanilide (AAPF) was used in this study [31]. Savinase can hydrolyse one bond in this tetrapeptide, therewith releasing the strongly yellow coloured nitroanilide that can easily be detected by visible light extinction. As the hydrolysis reaction follows Michaelis-Menten kinetics, the *initial* reaction rate depends linearly on the enzyme concentration as long as the tetrapeptide concentration is much higher than the enzyme concentration.

The sensitivity of this method is very high and Savinase concentrations as low as 0.1 μg ml^{-1} can be detected accurately. The other important advantage of this method is that the enzyme can be detected selectively in the presence of other proteins (e.g. protein fragments removed from the soiled cloth). However, the weak point of this type of concentration determination in a depletion experiment is that all the loss of enzyme activity during either the preparation of the solutions or the adsorption experiment itself will be detected as adsorption. This limits the experimental conditions to adsorption

measurements in case of low hydrolysis rates (in the low concentration and temperature ranges).

Experimental procedure

The visible light extinction of released nitro anilide was detected at 400 nm with a Perkin-Elmer Lambda 15 UV/VIS (double beam) spectrophotometer with thermostatted 4 ml glass cuvettes (optical path length is 1 cm). The solution in the cuvettes was constantly stirred with a small magnetic stirrer. The detailed experimental procedure is given in Appendix 2.4.

The crucial parameter for reproducible execution of this procedure proved to be the temperature during the preparation of the enzyme solution and during the reaction between the enzyme and the AAPF substrate in the cuvette. The enzyme solutions have to be cooled rigorously in order to prevent autolysis. The strong temperature dependence of the enzymatic reaction [32] requires the cuvettes to be thermostatted and to bring the buffer solution into thermal equilibrium before the AAPF and enzyme solutions are added.

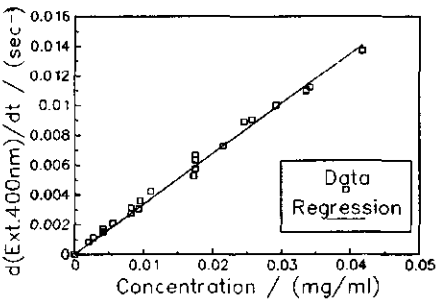

Figure 2.6 Enzymatic activity calibration curve of Savinase on AAPF.

The calibration curve (Figure 2.6) is based on Savinase concentrations determined with UV 280 nm extinction. The measurements relating to this curve were performed over three days using freshly isolated enzyme and newly prepared substrate solutions (see Appendix 2.4). The fact that these data points did not deviate from the average curve indicated that the reproducibility is satisfactory. The calibration curve can be represented by a linear function:

$$\Delta(\text{Extinction}_{400\ nm})/\Delta t = 0.35\ \text{ml mg}^{-1}\,\text{sec}^{-1}\cdot[\text{Savinase}]\ ;\ \text{with}\ 0<[\text{Sav}]<0.03\ \text{mg ml}^{-1}.$$

2.3.6 Determination of protein purity with Reversed Phase HPLC

Prior to the adsorption experiment both the Savinase and the PMS-Savinase samples were subjected to Reversed Phase HPLC to check for the presence of autolysis products. The method was previously reported [33] and is summarized in Appendix 2.5.

The binary gradient started with a hydrophilic eluent and ended with a more hydrophobic one. This type of gradient does not only give a separation but also an indication of the affinity of the fragments for a hydrophobic surface such as polystyrene. The proteins with the shortest retention time will be more hydrophilic and thus better soluble in water than those with longer retention times. This indicates that the short retention time fragments will probably have a lower affinity for the surface.

In order to prevent any autolysis during the preparation of the HPLC sample, the pH of the solution is lowered to pH≈2 by the addition of concentrated TFA (trifluor acetic acid). The detection is by UV 214 nm extinction and in the case of radio-active protein complemented by liquid scintillation counting (either continuous or by collecting fractions).

2.4 Results

2.4.1 RTT adsorption detection using "old labelled" PMS-Savinase

As will become clear in the following, the absolute values of the adsorption data reported in this section are not correct. Therefore discussion on the adsorption values will be brief and only the consequences of the detection method will be reported here.

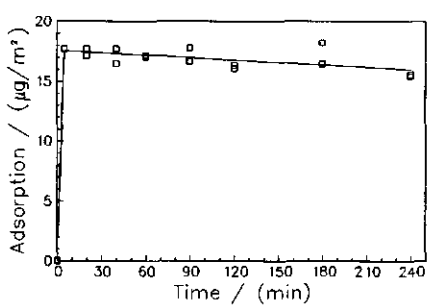

Figure 2.7 PMS-Savinase adsorption versus time. Detection: RTT with "old" labelled enzyme. Conditions: pH=10, T=30°C, C_e=3.5 μg/ml, S=0.17 m².

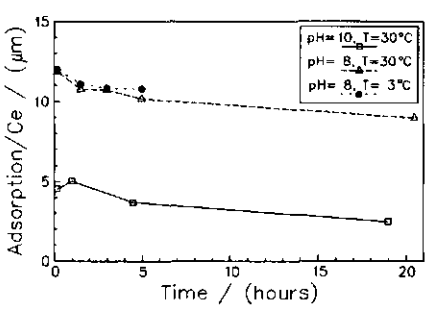

Figure 2.8 PMS-Savinase adsorption versus time at different pH en temperatures. Detection: RTT with "old" labelled enzyme. Conditions: C_e≈2 μg/ml, S=0.2 m².

Time dependence

The adsorption of PMS-Savinase on PS-latex (A) as a function of contact time is given in the Figures 2.7 and 2.8. The adsorbed amount (corrected for the equilibrium concentration by assuming a linear relation between Γ and C_e) decreased with increasing contact time. The decrease could be the result of autolysis of PMS-Savinase originating

from the residual activity. This was confirmed by the fact that decreasing the enzymatic activity, by lowering the pH and temperature, diminished the decrease in adsorption with time. Therefore all experiments described in this chapter were performed at 3°C and in most cases at pH=8.

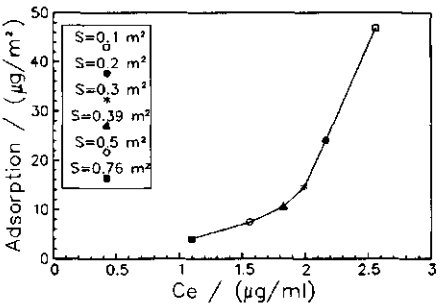

Figure 2.9 Adsorption isotherm of PMS-Savinase with varying surface areas. Detection: RTT with "old" labelled enzyme. Conditions: pH=8, contact time=90 min.

Surface area dependence

The adsorbed amount per unit area of a monodisperse species should be independent of the total surface area in contact with the solution and only be dependent on the equilibrium concentration. In order to check that the determined depletion values were caused by such a "normal" adsorption of protein on the polystyrene latex, the surface area was varied keeping the starting concentration (C_0) and all other experimental parameters constant. The results are shown in Figure 2.9. The adsorption isotherm increased progressively with C_e and differed significantly from the usual isotherm shape which is linear at low concentrations and approaches a plateau value at higher concentrations. In other words : the adsorption increased with a decrease of surface area more strongly than would be expected from the increase in equilibrium concentration. The first conclusion drawn from these results was that the measured amount could not be a "normal" adsorption.

Similar phenomena were described in the literature [34] for the adsorption of *polydisperse* polymers on solid surfaces. The reason behind this area dependence of the adsorption is easily understood when one keeps in mind that higher molecular mass (larger) polymers adsorb preferentially over lower molecular mass (smaller) ones [35]. For sake of simplicity we will consider a polydisperse solution containing only two polymers of different molecular mass. Both polymers will adsorb when a relatively large surface area is exposed and the surface coverage is less than 100%. However, reducing the surface area below the value needed for both polymers to adsorb independently leads to a replacement of small

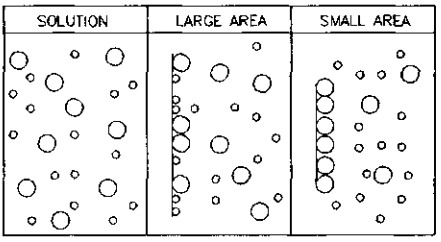

Figure 2.10 Simple representation of adsorption from polydisperse solutions.

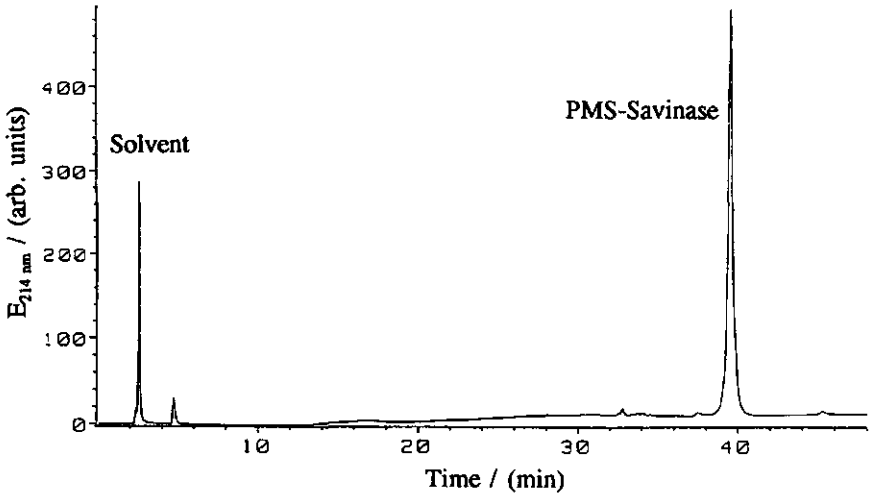

Figure 2.11a HPLC chromatogram of PMS-Savinase.

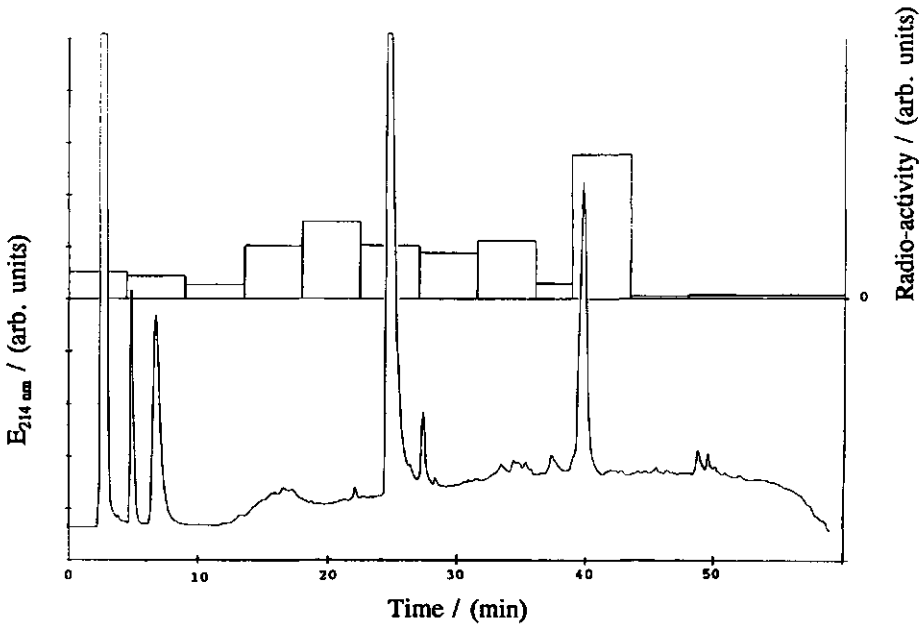

Figure 2.11b HPLC chromatogram of PMS-[^{3}H] Savinase (old labelling method).

polymers by large ones. The driving force behind this replacement is the decrease in free energy of the total system which follows directly from the preferential adsorption of the larger polymer. Such a replacement is visualized in Figure 2.10 and from this simple representation it is also clear that the adsorbed amount in mass per surface area increases when large polymers replace small ones. Therefore with polydisperse systems a decrease in surface area can lead to a sharp increase in adsorption.

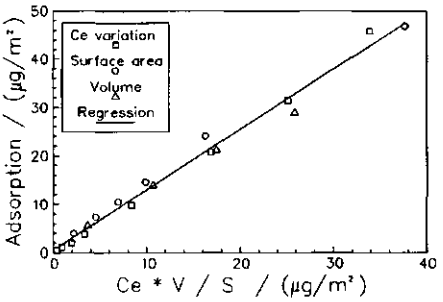

Figure 2.12 Adsorption of PMS-Savinase versus C_e*V/S. Detection: RTT with "old" labelled enzyme. Conditions: pH=8, contact time=90 min.

Cohen Stuart et al. [34] showed with an elegant mathematical argument that the adsorption from a polydisperse solution is a function of the equilibrium amount of polymer in solution per surface area ($C_e \cdot V/S$) and not of the equilibrium concentration (C_e) only, as is the case for monodisperse solutions. In order to determine whether the measured protein adsorption obeyed the dependence described for polydisperse polymer solutions quantitatively, the three parameters C_e, V and S where changed as independently as possible. The experimental setup caused changes in V and S to be accompanied by changes in C_e. Figure 2.12 shows that plotting the adsorption as a function of $C_e \cdot V/S$ resulted in *one*, linear, isotherm just as predicted for polydisperse solutions.

The remaining question was : where did the polydispersity that is so uncharacteristic for protein solutions originate from?

Polydispersity of radio-actively labelled Savinases and the consequences for adsorption measurement

The HPLC chromatograms of PMS-Savinase and Savinase, both unlabelled and labelled, are given in Figures 2.11 and 2.13, respectively. These chromatograms show clearly that the radio-actively labelled enzymes caused the polydispersity in the solutions whereas the unlabelled enzymes were reasonably monodisperse. For [^{3}H]-Savinase only 60% of the total radio-activity present was on the Savinase peak and for PMS-[^{3}H]-Savinase it was even worse : only 30% corresponded to the main peak. The impurities had shorter retention times and therefore they were more hydrophilic than the enzyme itself. The presence of these fragments was ascribed to autolysis during the labelling reaction and/or dialysis following that reaction.

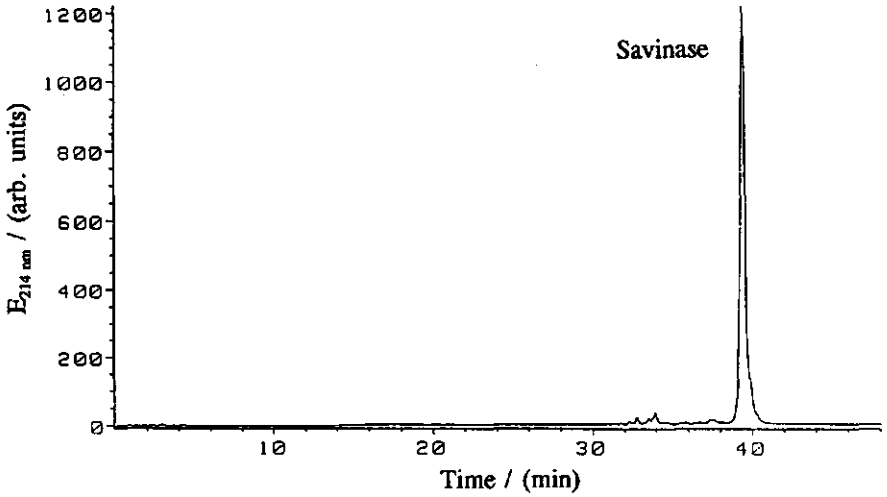

Figure 2.13a HPLC chromatogram of Savinase.

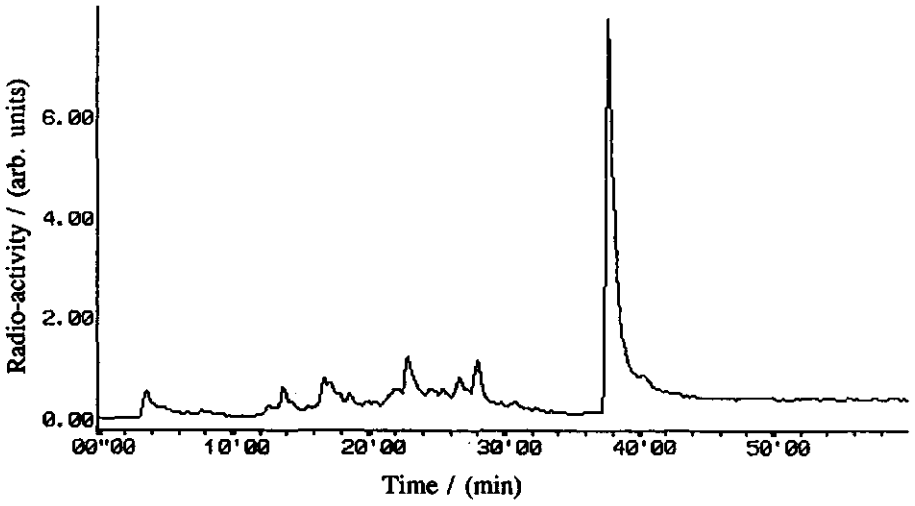

Figure 2.13b HPLC chromatograms of [^{3}H]-Savinase (old labelling method).

The fact that only the radio-actively labelled enzyme was partly degraded caused the failure of the radio-active tracer detection technique. The above mentioned implicit demand for equality between labelled and unlabelled enzyme was obviously not met. The results showed that the labelled enzyme was on average more hydrophilic and therefore better soluble in water than the unlabelled one. The *measured* adsorption was lower than the *actual* adsorbed amount as only the depletion of *radio-actively labelled* enzyme was measured. This explained why the *detected* adsorption was much lower than that of most proteins studied in the literature.

As mentioned above, polydispersity can only cause an area dependence when the surface is (nearly) covered. The results shown in Figure 2.12 suggest that even at low Savinase concentrations the surface was almost completely covered with both Savinase and autolytic fragments. Although the presence of the fragments explained the existence of an area dependence it was still not obvious why the detected adsorbed amount *increased* with decreasing surface area. The fact that the detected adsorbed amount was lower than the actual adsorption indicated that unlabelled enzyme adsorbed preferentially. Therefore one would expect that the detected adsorption would decrease with decreasing surface area. One of the possible causes of this unexpected increase could be the fact that the fragments occupied more surface area per unit mass than the complete Savinase so that desorption of these fragments increased the adsorbed amount. However, without further investigation of the adsorption characteristics of the autolytic fragments of the labelled enzyme we can not prove this to be the explanation of the phenomenon.

2.4.2 RTT adsorption detection using "new" labelled Savinases

Adsorption of PMS-Savinase

The HPLC chromatograms of the three PMS-[^{3}H]-Savinase batches are shown in Figures 2.14 to 2.16. Comparison with the chromatogram of the PMS-[^{3}H]-Savinase made according to the "old" labelling procedure (see Figure 2.11b) shows that the purity of the labelled enzyme is improved significantly by using the new procedure.

The isotherms of PMS-Savinase measured with these different batches are shown in Figure 2.17. The adsorption isotherms measured with batch 8-'90 and 11-'90 showed a "high affinity" character i.e. a sharp increase in adsorption at low equilibrium concentrations. At relatively low concentrations a plateau value of 3 mg m^{-2} is reached which corresponds to a monolayer coverage of the surface (see also chapter 3). The adsorption detected with the "8-'90" batch showed hardly any surface area dependence and was independent of the contact time (15 min to 2 hours). The sequence of addition of enzyme and polystyrene surface did not influence the adsorption value.

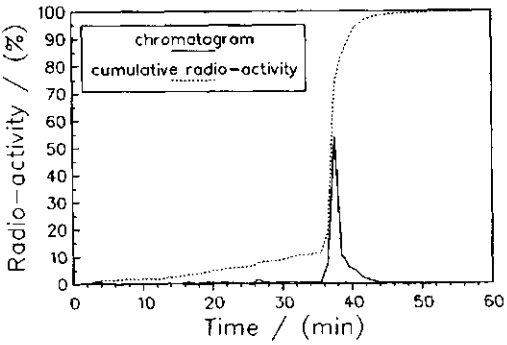

Figure 2.14 HPLC chromatogram of PMS-[^{3}H]-Savinase: batch 8-'90 (new labelling method).

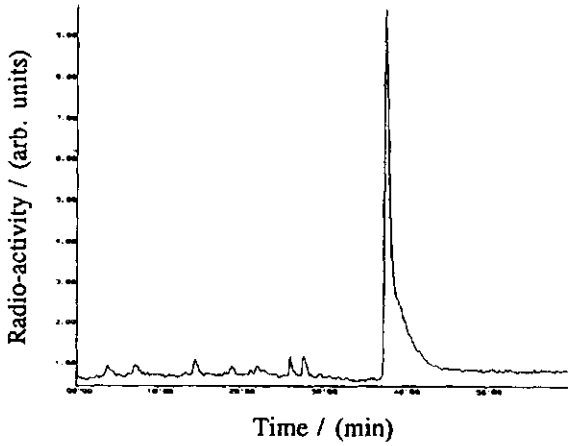

Figure 2.15 HPLC chromatogram of PMS-[^{3}H]-Savinase: batch 11-'90 (new labelling method).

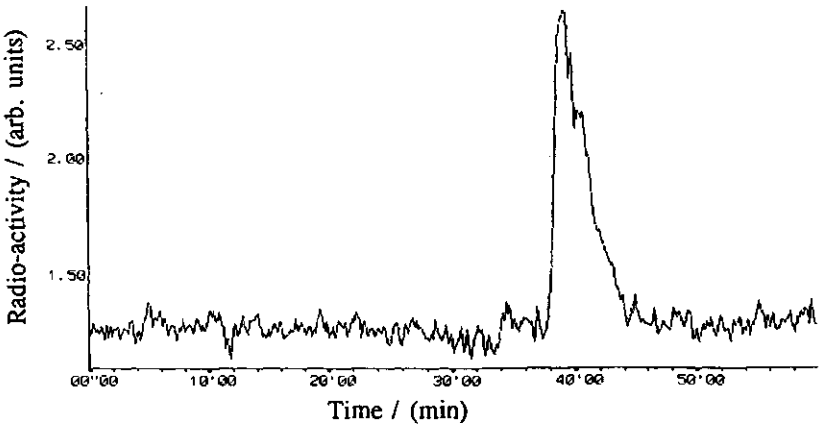

Figure 2.16 HPLC chromatogram of PMS-[^{3}H]-Savinase: batch 3-'91 (new labelling method).

The isotherms show that the *detected* amount of adsorbed enzyme increased drastically with improvement of the purity of the radio-actively labelled protein. In Table 2.2 the data obtained with "old" and "new" labelled PMS-[^{3}H]-Savinase under similar experimental conditions are compared. This comparison shows that at low concentrations the *detected* adsorbed amount was increased by a factor of 20! It can therefore be concluded that the previously determined low PMS-Savinase adsorption was caused by polydispersity of the labelled enzyme.

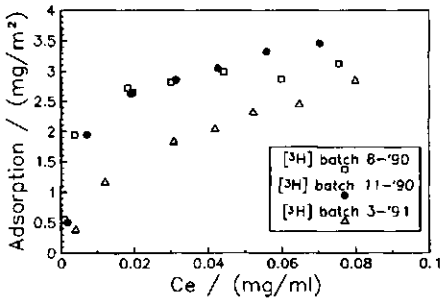

Figure 2.17 Adsorption isotherms of PMS-Savinase determined with several batches of "new" labelled enzyme. Conditions: pH=8, data points are average of three contact times (30, 60 and 90 minutes).

The differences shown in Figure 2.17 can be understood when we look in more detail at the corresponding HPLC chromatograms. Two differences could be distinguished between the chromatograms of the differently labelled batches and between those of labelled and unlabelled enzyme. The first difference was the amount of hydrophilic components present (with retention times shorter than Savinase) and the second was the existence of

Table 2.2 Comparison of the adsorption at pH=8 detected with "old" and "new" labelled PMS-Savinase.

RTT detection	C_e (mg ml^{-1})	Adsorption (mg m^{-2})	Surface area (m^2)
"old" label	0.0026[a]	0.0469	0.1
"new" label	0.0026[b]	1.3261	0.02
"new" label	0.0011[a]	0.5549	0.02
"new" label	0.0038[a]	1.9430	0.02

[a] Experimental data points.
[b] Calculated by interpolation of data points given below.

a hydrophobic "tail" linked with the Savinase peak. The presence of hydrophilic labelled protein is expected to decrease the slope of the isotherm at low concentrations. This is logical because the hydrophilic components are better soluble in water and the presence of labelled material in solution will increase the determined equilibrium concentration and hence decrease the amount of adsorbed enzyme as it is calculated.

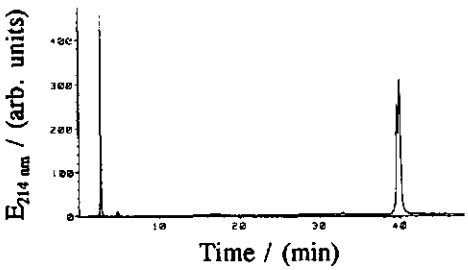

Figure 2.18a HPLC chromatogram of PMS-Savinase batch A.

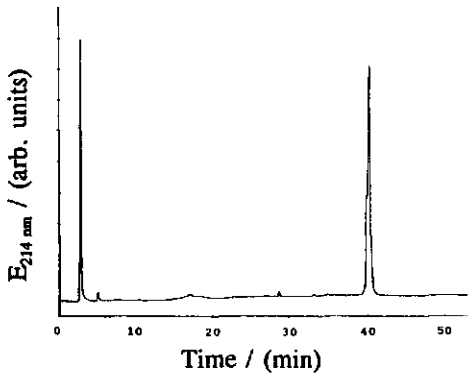

Figure 2.18b HPLC chromatogram of PMS-Savinase batch B.

The second difference was the "tail" on the Savinase peak that was detected in all radioactively labelled enzymes while it was absent in the unlabelled protease. A larger "tail" resulted in an isotherm that continued to rise slightly at higher concentration instead of reaching a plateau value. This could be explained by assumption that the more hydrophobic "tail" components of the labelled enzyme adsorbed preferentially over the unlabelled PMS-Savinase. After a monolayer of labelled and unlabelled enzyme covered the surface, a further increase in equilibrium concentration resulted in a replacement of unlabelled by preferentially adsorbing labelled "tail" material. The increase in labelled material on the surface is interpreted as an increase in adsorbed amount.

These differences showed that the radioactive tracer technique was still not giving reliable results for the adsorption of PMS-Savinase. The labelling method was improved strongly compared to but it was not reproducible (e.g. batch 3-'91 resulted in a lower affinity isotherm). The best results were obtained with the "8-'90" batch as could be concluded from both the HPLC chromatogram and the shape of the isotherm.

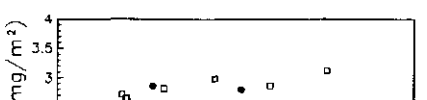

Figure 2.19 Comparison of different batches unlabelled PMS-Savinase at pH=8. Detection: RTT with "new" labelled enzyme (batch 8-'90).

Although only the labelled enzyme is detected the quality of the *unlabelled* PMS-Savinase could influence the results as well. Therefore the HPLC chromatograms of various batches of unlabelled PMS-Savinase were measured. The largest difference seen

up to now is shown by comparison of Figures 2.18a and b. Batch A (Figure 2.18a) shows a clear "split" in the Savinase peak indicating the presence of at least two components. These could be two different conformations of Savinase or the complete protease and Savinase that missed a short string of amino acids. However, the presence of the "split" in the HPLC chromatogram did not result in different adsorption values (Figure 2.19), apparently because the hydrophobicity of the two species is very similar.

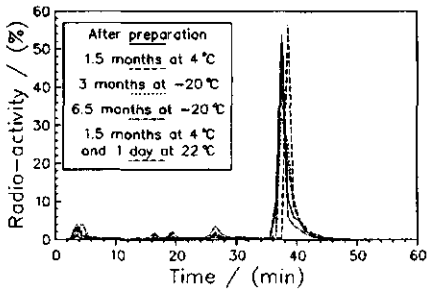

Figure 2.20a Normalized HPLC chromatogram of PMS-[^{3}H]-Savinase (batch 8-'90) after different storage times and conditions.

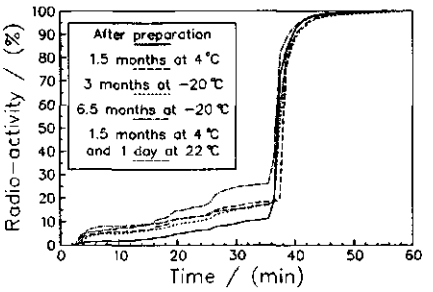

Figure 2.20b Cumulative of detected radioactivity after HPLC-separation of PMS-[^{3}H]-Savinase (batch 8-'90) after different storage times and conditions.

Storage stability of labelled PMS-Savinase

The HPLC chromatograms of the "8-'90" batch PMS-[^{3}H]-Savinase (Figures 2.20) indicate that a slow degradation occurs even at low pH (pH $\approx$ 5) and T = −20°C. The rate of increase of the total amount of radioactive material with shorter retention times than that of the enzyme is diminished by a decrease in temperature as can be concluded from comparison with the chromatogram of a sample left at room temperature for a day.

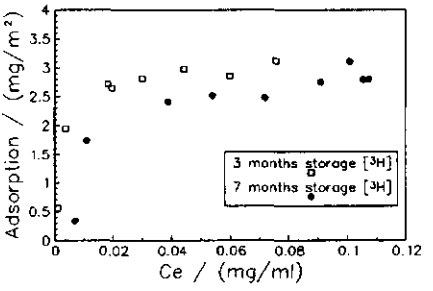

Figure 2.21 Effect of storage of PMS-[^{3}H]-Savinase on adsorption. Detection with "new" labelled enzyme (batch 8-'90). Conditions: pH = 8.

At −20°C the degradation seems to level off after the first months and the total amount of hydrophilic material is about the same after 3 and 6.5 months. However, the fraction with very short retention times increased in this period. The adsorption isotherms measured with these two samples are shown in Figure 2.21. The small differences in HPLC chromatograms resulted in significantly different *detected* adsorbed amounts although the shapes of the isotherms were much the same.

2.4.3 Adsorption measurement with UV 280 nm detection

Influence of PS-particles in the supernatant

An accurate detection of the protein concentration is only possible in the absence of other UV-absorbing components in solution. In these adsorption experiments the presence of small amounts of polystyrene latex particles hampered the determination of the equilibrium concentration. There are in principle two ways to decrease the amount of PS-particles in solution. The easier is by increasing the duration of the centrifugation from 10 to 30 minutes but this did not decrease the scattering of light significantly. This is probably caused by the fact that the PS-particles are easily redispersed into the liquid when the centrifugation is stopped or when the samples are handled after centrifugation.

Filtration of the supernatant over a 0.2 μm filter is an alternative used in the literature [36]. It reduced the light scattering but a considerable adsorption of PMS-Savinase on the filters made it impossible to use this method in our study. It is interesting to note that large differences occur for the adsorption on different brands of filters. At a concentration of 0.2 mg ml^{-1} and volume of 1 ml the Millex-GV® (0.22 μm filter unit no.SLGV 025 BS, ex Millipore) filters caused losses of up to 85% while the Acrodisc® (0.2 μm filter unit no.4192, ex Gelman Sciences) filters only about 5% (both filters were specified as low protein binding filters).

In order to reduce light scattering as much as possible only a small sample of the supernatant was used for the UV extinction measurement. Unfortunately, relatively large variations in scattered light could not be prevented.

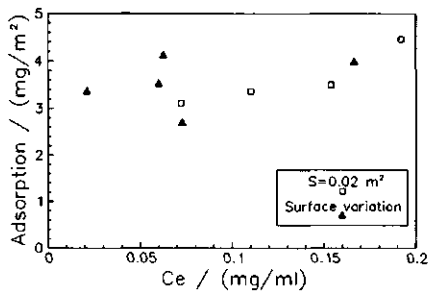

Figure 2.22a Adsorption isotherm of PMS-Savinase (batch A) detected with UV 280 nm extinction. Conditions: pH=8, t_c=60 min, V=1 ml.

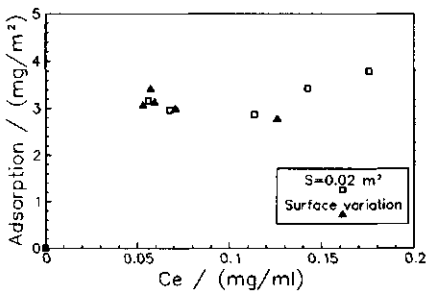

Figure 2.22b Adsorption isotherm of PMS-Savinase (batch B) detected with UV 280 nm extinction. Conditions: pH=8, t_c=60 min, V=1 ml.

Adsorption isotherms

Adsorption isotherms were determined using this detection method under various experimental conditions. The adsorption of two different batches of PMS-Savinase at pH=8 is shown in Figures 2.22a and b. The adsorption shows no significant dependence on either a variation of surface area or a difference between batches of PMS-Savinase (see also HPLC chromatograms in Figure 2.18a and b).

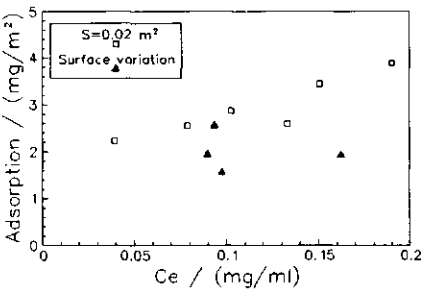

Figure 2.23 Adsorption isotherm of PMS-Savinase (batch B) detected with UV 280 nm extinction. Conditions: pH=10, t_c=60 min, V=1 ml.

In Figure 2.23 the isotherm at pH=10 is shown while the ionic strength and temperature were kept constant at 0.01 M and 3°C respectively. The relatively large variations caused by the presence of PS-particles made it impossible to draw definite conclusions about small differences in adsorption as those between pH=8 and pH=10.

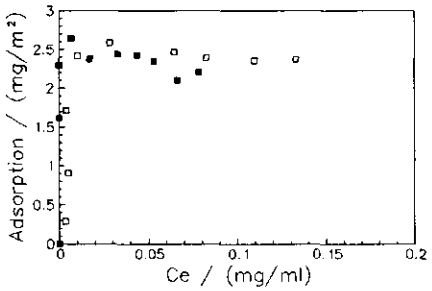

Figure 2.24 Reproducibility of the adsorption isotherm of PMS-Savinase. Conditions: Lowry colouring, pH=8, t_c=60 min, S=0.02 m^2 and 0.04 m^2.

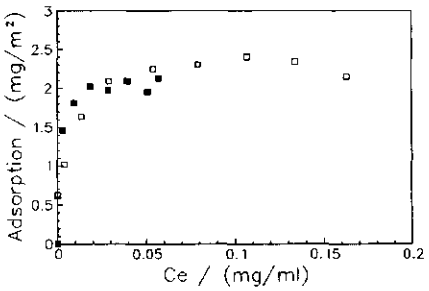

Figure 2.25 Reproducibility of the adsorption isotherm of PMS-Savinase. Conditions: Lowry colouring, pH=10, t_c=60 min, S=0.02 m^2 and 0.04 m^2.

2.4.4 Adsorption measurement with Lowry colouring method

The experimental isotherms at pH=8 and pH=10 are presented in Figure 2.24 and 2.25 respectively. The isotherm at pH=8 has a high affinity character and the one at pH=10 shows a somewhat lower affinity. The plateau value corresponds to a monolayer of enzyme. In both figures the data of two independent measurements are given and the reproducibility proves to be good. The adsorption is independent of the contact time in

the range from 15 minutes to 2 hours (Figure 2.26). Figure 2.27 shows that no significant surface area dependence can be detected.

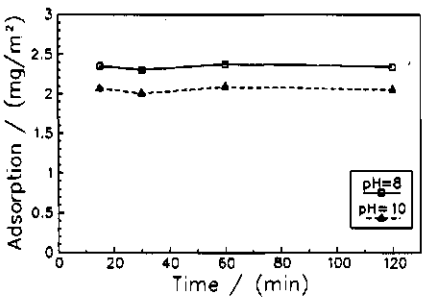

Figure 2.26 Time dependence of PMS-Savinase adsorption. Conditions: Lowry colouring, $C_e \approx 0.07$ mg/ml, S=0.04 m².

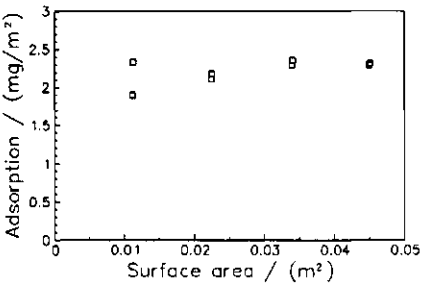

Figure 2.27 Surface dependence of PMS-Savinase adsorption. Conditions: Lowry colouring, pH=8, $C_e > 0.07$ mg/ml.

Influence of enzymatic activity on Lowry colouring

During the *second* step of the Lowry colouring reaction the Cu^+ has to react with the Folin reagent at pH ≈ 10 and a temperature of 25°C over 1 hour. As these conditions are optimal for the enzymatic activity of Savinase, the influence of possible autolytic activity of the protease on the colouring reaction was investigated. Samples were taken after various times in the second step of the Lowry colouring reaction showed only a residual enzymatic activity of 0.5%. The absence of enzymatic activity could be explained by an irreversible loss of activity of the enzyme in the alkaline copper sulphate solution (pH ≈ 13) that is added in the *first* reaction step.

No influence of residual activity of PMS-Savinase on the protein concentration determination is therefore expected. We used this result also to justify the use of a Lowry calibration curve of active Savinase to determine the concentration of PMS-Savinase.

Influence of the pH of the protein solution on the Lowry colouring

The results of two Savinase concentration determinations (0.1 and 0.25 mg ml^{-1}) in a pH-range between 7 and 11 are collected in Table 2.3. No significant pH-dependence of the Lowry colouring method could be detected.

Statistical reproducibility of adsorption measurement with Lowry detection

In order to analyze the factors that determine the reproducibility of the detection method it is necessary to know how the adsorption is calculated from the original measurements. This is shown in detail in Appendix 2.6 together with the derivation of the standard deviation of the adsorption.

Table 2.3 Influence of pH of protein solution on Lowry colouring.

pH	Detected concentration Solution 1 (mg ml^{-1})	Detected concentration Solution 2 (mg ml^{-1})
7	0.0977	0.2645
8	0.0972	0.2582
10	0.0981	0.2506
11	0.1007	0.2655

Using equation (2.6.4) the standard deviation can be determined for a typical adsorption experiment and the result is surprising. The standard deviation of adsorption is about 0.6 mg m^{-2} when the mass of added enzyme solution is 1 g, the volume of the aqueous phase is 1.5 ml, the surface area is 0.045 m^2 and the standard deviation (σ) of the concentration determination is about 0.015 mg ml^{-1} (Figure 2.5). This means that a typical adsorption of 2.5 mg m^{-2} would have a 95% confidence limit of 2.5 $\pm$ 1.2 mg m^{-2} ! The reproducibility based on the experimental calibration curve is much less than the experimentally determined one. A possible explanation for this phenomenon is that the calibration curve might be better (smaller σ) than we have calculated it here. This could be checked by reproducing the calibration curve with more data points.

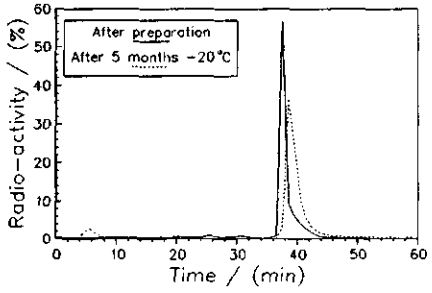

Figure 2.28a Normalized HPLC chromatogram of [^{3}H]-Savinase after different storage times (batch 8-'90, new labelling method).

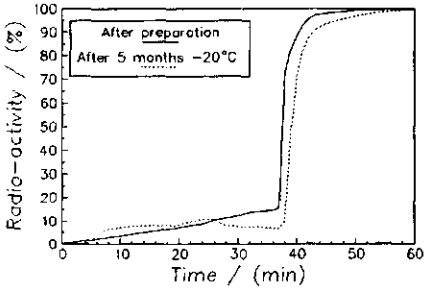

Figure 2.28b Cumulation of detected radioactivity after HPLC-separation of [^{3}H]-Savinase (batch 8-'90) after different storage times.

2.4.5 Adsorption of active Savinase measured with RTT and Lowry colouring

The HPLC chromatogram of the [^{3}H]-Savinase is shown in Figure 2.28 and the measured isotherms are given in Figure 2.29. The chromatogram shows that hardly any hydrophilic

components were present in the [^{3}H]-Savinase and that the hydrophobic "tail" was small compared with that of PMS-[^{3}H]-Savinase. The isotherm is of high affinity and reaches a plateau value of about 2.5 mg m^{-2}. The isotherms measured with RTT and Lowry colouring method do not differ significantly.

The conclusion from the HPLC chromatogram and the comparison of adsorption isotherms is that the labelled active Savinase compares better with the unlabelled enzyme than is the case for PMS-Savinase. This means that this batch of labelled *active* Savinase, made according to the improved labelling procedure, could be used for reliable adsorption measurement with the RTT method. The HPLC chromatogram of [^{3}H]-Savinase that was stored for 5 months (Figure 2.28) showed that there was hardly an increase of radio-activity at shorter retention times (the difference in retention times of the mean peak is caused by a small difference in the way the fraction were collected after separation on the HPLC column). It can therefore be concluded that [^{3}H]-Savinase is more stable than PMS-[^{3}H]-Savinase. This indicates that the residual activity of PMS-Savinase did not lead to less degradation in solution.

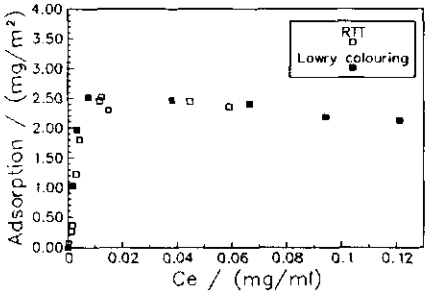

Figure 2.29 Comparison of Savinase adsorption detected with RTT (batch 8-'90) and Lowry colouring method: pH=8, t_c=30 min (RTT) and average of 15, 30 and 60 minutes (Lowry).

2.4.6 Adsorption of active Savinase measured with the AAPF concentration determination method

The reliability of the AAPF method was verified by comparison of the adsorption values with above reported isotherms detected with the Lowry colouring method. Two concentration ranges were investigated since the loss of enzymatic activity caused by (enzyme concentration dependent) autolysis was expected to influence the results. In Figure 2.30 it can be seen that the adsorption values measured with Lowry and AAPF do not differ significantly at equilibrium concentrations below 0.03 mg ml^{-1}. However, at higher C_e values the AAPF method results in a higher measured adsorbed amount as was expected (Figure 2.31). At concentrations higher than 0.03 mg mg^{-1} the Lowry colouring method is preferred but at low enzyme concentrations the AAPF method is reliable. The high sensitivity of the method makes it very useful for the determination of the rising part of the adsorption isotherm.

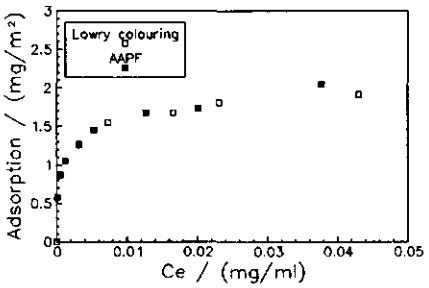

Figure 2.30 Comparison between Savinase isotherms detected with AAPF and Lowry colouring method. Conditions: pH=8, I=0.05 M, S=0.03 m².

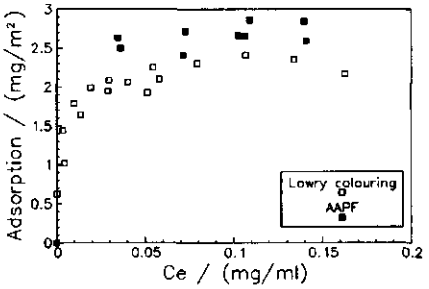

Figure 2.31 Comparison between Savinase isotherms detected with AAPF and Lowry colouring method. Conditions: pH=10, I=0.01 M, S=0.045 m².

In order to be applicable to the concentration determination of Savinase in the presence of other proteins and protein fragments the AAPF method should not be influenced by the presence of other proteins in the enzyme solution. In principle the protein could function as a substrate and compete with the AAPF for the enzyme. This would result in an apparently too low enzyme concentration because the calibration curve is measured in the absence of any other protein in solution. We verified the influence of BSA on the AAPF method by comparing the proteolytic activity of samples from a Savinase solution with and without added BSA. As shown in Figure 2.32 the enzymatic activity detected with the AAPF method immediately after addition of Savinase (t=0) is identical in both solutions. Even the presence of hydrolysed BSA (after longer reaction times) did not effect the AAPF concentration determination. However, the presence of BSA and its hydrolysed fragments did have an unexpected but logical effect: it decreased the loss of enzymatic activity caused by autolysis that occurred in a "clean" Savinase solution. This indicates that although we have to choose "low-enzymatic-conditions" to prevent autolysis in "clean" Savinase solutions we could use the AAPF method to measure the adsorption on protein soiled surfaces under conditions more favourable for hydrolysis.

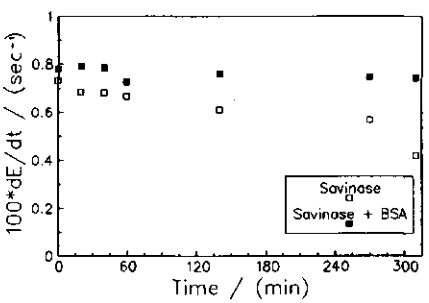

Figure 2.32 Detected enzymatic activity from Savinase solutions with and without BSA as a function of time. Conditions: pH=10, I=0.01 M, room temperature, [Sav]=0.02 mg/ml, [BSA]=2.2 μg/ml.

2.5 Conclusions

The radio-actively labelled Savinase made according to [24] was degraded by autolysis during the labelling reaction and following dialysis. The polydispersity of the labelled enzyme caused the failure of RTT detection method and resulted in a *detected* adsorption that was a factor of 10 lower than the *actual* adsorption of Savinase. The adsorption from the polydisperse enzyme solution depended linearly on the volume of the enzyme solution and was inversely proportional to the surface area in a manner similar to that described by Cohen Stuart et al [34] for polydisperse polymer solutions.

Changes of the conditions of the labelling reaction and dialysis improved the purity of the labelled enzyme. The use of this labelled enzyme resulted in much more reliable adsorption measurements. However, the differences between the various batches of labelled PMS-Savinase rendered this method unfit for reproducible measurements.

The UV 280 nm absorption method has a high detection limit making the method unsuitable for detection of the rising part of the isotherm. The UV 280 nm detection of Savinase adsorption on PS-latex was hampered by the scattering of light by small PS-particles still present after centrifugation.

The Lowry colouring concentration determination proved to be an reliable and reproducible method for the measurement of the adsorption of active and PMSF-inhibited Savinase on PS-latices. However, this method can not be used for the detection of adsorption on protein-covered surfaces as it detects all proteins present in solution. The detection limit of the method reported here is about 0.02 mg ml^{-1} and this is too high for the accurate determination of the rising part of the adsorption isotherms.

The concentration determination of active Savinase on the soluble substrate AAPF gave reliable and reproducible results in the range between 0.1 μg ml^{-1} and 30 μg ml^{-1}. Therefore the method is suitable for measurement of the rising parts of the isotherms. As the proteolytic enzyme is detected selectively, the method can also be used to detect Savinase in a mixture of other proteins (e.g. protein fragments removed from a protein covered surface). Disadvantages of this method are that only active Savinase can be detected and that all loss of enzymatic activity (including that caused by autolysis) is attributed to adsorption.

2.6 References

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APPENDIX 2.1

INHIBITION OF SAVINASE WITH PMSF

Purification

1) The Savinase was isolated from a commercial preparation (ex NOVO-Nordisk) and it was purified using chromatographic procedures (section 2.2.1) by Bio- and Nutrition Sciences Department (URL Vlaardingen). The purified Savinase (batch 90.1), concentration of about 83 mg ml^{-1}, is stabilized by 30% glycerol/acetate pH=5 and stored at −20°C.

Isolation

2) Separate the Savinase from glycerol and acetate-buffer solution by gel filtration, using a PD-10 column. First eluate the column with 25 ml 0.05 M KH_2PO_4 pH=7 buffer then bring 1 ml Savinase 90.1 solution on the column. When the sample has run into the column add 1.5 ml of the phosphate buffer on the column.
3) When all the buffer is run in the column, elute the Savinase with 1.2 ml of the phosphate buffer and dilute to 50 ml in the phosphate buffer.
4) Determine the Savinase concentration with UV 280.

Inhibition

5) Prepare 3 litres 0.6 μM Savinase solution by dilution with the phosphate buffer.
6) Measure the enzyme activity with the AAPF method and record an UV spectrum between 240 and 340 nm.
7) Add 4 ml 0.5 M PMSF (phenylmethanesulfonyl fluoride) in 2-propanol solution to the enzyme solution and stir the solution for about 30 minutes at ambient temperature. Check/adjust pH=7.
8) Repeat step 6 to determine and check the residual activity and changes in the UV spectrum.
9) Dialyse the solution against demineralized water for at least 24 hours at 4°C.
10) Adjust the pH of the solution to 6<pH<7 with 1 M HCl while stirring rigorously and repeat step 6. Ensure that all free PMSF is removed.
11) Concentrate the PMS-Savinase solution by ultrafiltration over a YM10 membrane (ex Amicon; no. 13642) and thereafter over an Acrodisc 0.2 μm filter unit.
12) Repeat step 6 to determine and check the residual activity and changes in the UV spectrum. Reversed Phase HPLC should also be carried out to check if autolysis fragments remained in the final solution.

Storage

13) Divide and store the PMS-Savinase solution in 2 ml Eppendorf micro test tubes at −20°C until use.

APPENDIX 2.2

RADIO-ACTIVE LABELLING OF SAVINASE AND PMS-SAVINASE

Isolation

1) Separate the Savinase (batch 90.1, see Appendix 2.1) from glycerol and acetate-buffer solution by gel filtration, using a PD-10 column. First eluate the column with 25 ml 0.05 M KH_2PO_4 pH=7 buffer. Add 0.5 ml Savinase 90.1 solution to the column. When the sample has run into the column add 2.0 ml of the phosphate buffer.
2) When all the buffer has run into the column, eluate the Savinase with 0.7 ml of the phosphate buffer.
3) Determine the Savinase concentration with UV 280.
4) Dilute to a Savinase concentration of about 15 mg ml^{-1} with the phosphate buffer.

Protein labelling

5) Dissolve the tritiated $NaCNBH_3$ (10 mCi) by adding 100 μl phosphate buffer in the ampoule. Add 100 μl Savinase solution.
6) Start the methylation by adding 15 μl formaldehyde and stir the solution for 2½ hours at ambient temperature.

PMS-[^{3}H]-Savinase route

7) Prepare 300 ml 3 mM PMSF by adding the PMSF to the phosphate buffer. Place 6 ml of the PMSF solution in a dialysis tube (cut-off 10 kDa). Take 100 μl solution out of the ampoule and add this to a dialysis tube. Close the tube.
8) Place the dialysis tube in 250 ml of the 3 mM PMSF solution and dialyse under gently stirring for 45 minutes at ambient temperature. Take a sample of the outer solution and determine the radio-activity.
9) Place the dialysis tube in 2 dm^3 5 mM acetate buffer pH=5 and dialyse over 48 hours at 4°C. Change solutions regularly (7 times at the least) and determine the radio-activity of every solution. The latter is done in order to check the removal of all free radio-activity (both radio-active water and labelled protein fragments).

[^{3}H]-Savinase route

7) Add 6 ml 5 mM acetate buffer pH=5 to a dialysis tube. Further add 100 μl solution out of the ampoule.
8) Place the dialysis tube in 2 litres 5 mM acetate buffer pH=5 and dialyse over 48 hours at 4°C. Change solutions regularly (7 times at the least) and determine the radio-activity of every solution. The latter is done in order to check if nearly all free radio-activity is removed.

Storage and analysis

10) Transfer the contents of the dialysis tube into separate 2 ml Eppendorf micro test tubes. Store the solutions at −20°C.
11) Reversed Phase HPLC should also be carried out to check if autolysis fragments remain in the final solution.

APPENDIX 2.3

PROCEDURE FOR PROTEIN DETERMINATION

WITH LOWRY COLOURING METHOD

Materials

Solution 1 : 2% Na_2CO_3 in 0.1 N NaOH
Solution 2 : 2.7% K-Na-tartrate ($C_4H_4KNaO_6.4\ H_2O$)
Solution 3 : 1% $CuSO_4$

Copper reagent : (should be prepared prior to use)
- 1 ml of solution 2
- 1 ml of solution 3
- 98 ml of solution 1

Folin reagent :
- 40 ml Folin reagent (ex Merck)
- 60 ml Millipore-Super-Q water

Procedure

1. Add 5 ml copper reagent to 1 ml of protein solution.
2. After 10 minutes 0.5 ml Folin reagent is added and mixed **RAPIDLY**.
3. The reaction tubes are placed in a water bath of 25°C for 1 hour.
4. The extinction at 750 nm is measured in a 1 cm path length glass cuvette.
5. Concentration determination by comparison of extinction value with calibration curve.

APPENDIX 2.4

PROCEDURE FOR THE DETECTION OF ENZYMATIC ACTIVITY ON AAPF

Equipment

The equipment used for time dependent extinction measurement of the nitro anilide that is released from the AAPF is a Perkin Elmer Lambda 15 UV/VIS spectrophotometer with thermostatted cuvette holders and magnetic micro-stirrers (Rank Bros Ltd.) and 4 ml glass cuvettes (optical path length is 1 cm).

Chemicals

* A 0.01 M, pH=9 TRIS buffer is prepared prior to use from a stock solution of 0.1 M TRIS that is kept at 4°C.
* A 6 mM AAPF solution is prepared prior to use by dilution of a stock solution of 150 mM AAPF in dimethylsulphoxide (DMSO) with 0.01 M, pH=9 TRIS buffer. During the day the solution is kept at 0°C.

Measurement procedure

1. The thermostat bath is adjusted to keep the temperature in the middle of a 4 ml glass cuvette filled with TRIS buffer at 25 $\pm$ 0.2°C.
2. The glass cuvettes in the measurement and the reference cell are filled with 2.3 ml TRIS buffer (0.01 M) and closed until the temperature is 25 $\pm$ 0.2°C.
3. A magnetic stirrer is added to the cuvette in the measurement cell.
4. 0.1 ml of the 6 mM AAPF solution is added and the measured extinction at 400 nm is set to zero (auto-zero of the apparatus).
5. Immediately thereafter 0.1 ml of the enzyme solution (of which the concentration C is to be determined) is added and the extinction measurement at 400 nm is started.
6. The extinction at 400 nm is measured as a function of time for 3 minutes.
7. The slope of the extinction versus time curve is determined between t=5 and t=20 seconds. For Savinase concentrations up to 0.04 mg ml^{-1} this time period is short enough to result in linear time dependencies. After longer reaction times and higher concentrations the slope diminishes as the substrate concentration decreases significantly with time.
8. Comparison of the slope with the calibration curve (Figure 2.6) determines the concentration C in the enzyme solution.

APPENDIX 2.5

REVERSED PHASE HPLC FOR

THE DETERMINATION OF PROTEIN PURITY

Column: Bakerbond 7116-00, Wide-Pore Butyl (C_4), 5 μm, 4.6*250 mm
Eluent: A = 0.06% TFA (trifluor acetic acid) in H_2O
B = 0.054% TFA in acetonitrile/water (80%/20%)
Flow rate: 1 ml min^{-1}
Sample loop: 100 μl
Detector: UV: 214 nm
Radio-activity: Ramona Radio HPLC detector type 5 LS ex Raytest.
Fraction lead through detector: 10%

Table 2.4 Binary gradient used for HPLC protein purity determination.

time (min)	% A	% B	Approximate relative dielectric constant
0	100	0	80
5	100	0	80
50	30	70	50
55	0	100	37
60	100	0	80

APPENDIX 2.6

VARIATION CALCULATION OF THE ADSORPTION MEASUREMENT WITH THE LOWRY COLOURING METHOD

Experimentally the main equation is:

$$\Gamma = (C'_0 * M_E - C_e * V_v)/S \qquad (1)$$

with Γ the adsorbed amount, C'_0 the enzyme concentration at the start of the experiment, M_E the mass of the added enzyme solution, C_e the equilibrium concentration, V_v the volume of the aqueous phase and S the total surface area in contact with the solution.

The various parameters as derived from experimental measurements:

1. $C'_0 = f(A_0 - A_{0,b})$ (concentration calculation via calibration curve)

2. $C_e = f(A_e - A_{e,b})$ (concentration calculation via calibration curve)

3. $V_v = M_E + M_{PS} * (1 - V_{PS})$ (total volume without PS-particles)

   with $V_{PS} = C_{PS} / \rho$ (volume PS-particles)

   with $C_{PS} = (M_{stock\text{-}PS} / M_{dilut\text{-}PS}) * C_{PS\text{-}stock}$ (mass fraction of PS in diluted PS-solution)

   with $C_{PS\text{-}stock} = (M_{PS+vial} - M_{vial})_{dry} / (M_{PS+vial} - M_{vial})_{sol}$

4. $S = M_{PS} *$ Surface density$_{PS}$ (total surface area)

   with Surface density$_{PS} = [C_{PS} / \rho] * 6\,10^{-6} / (D_{3,2}\,10^{-9})$

   with $C_{PS} = (M_{stock\text{-}PS} / M_{dilut\text{-}PS}) * C_{PS\text{-}stock}$ (mass fraction of PS in diluted PS-solution)

   with $C_{PS\text{-}stock} = (M_{PS+vial} - M_{vial})_{dry} / (M_{PS+vial} - M_{vial})_{sol}$

Experimentally determined parameters:

M (mass)	value ± 0.0001 g
A (extinction of light)	value ± 0.001 units
$D_{3,2}$ (diameter for surface area calculation)	value ± 25 nm

It can easily be shown that the contribution to the variance of Γ of the latter two parameters is not important when compared with both concentration determinations. The equation for the variation in Γ is given in (2).

$$var(\Gamma) = (\partial\Gamma/\partial C_0)^2 \cdot var(C_0) + (\partial\Gamma/\partial C_e)^2 \cdot var(C_e) \qquad (2)$$

In Figure 2.5 the prediction interval for a new observation is shown as calculated with linear regression [1,2]. The standard deviation (σ) of the concentration that is calculated from a measured extinction appears to be almost constant within the range of the experimentally determined calibration curve. The variance of the calculated adsorption is then:

$$var(\Gamma) = \sigma^2/S^2 \cdot (M_E^2 + V_v^2) \qquad (3)$$

The standard deviation of the adsorption is:

$$S_\Gamma = \sqrt{(var(\Gamma)} \quad \Leftrightarrow$$

$$S_\Gamma = \sigma\sqrt{(M_E^2 + V_v^2)}/S \qquad (4)$$

This general equation (4) assists in choosing the best experimental setup because it makes clear that the reproducibility of the adsorption is improved by

* increasing the accuracy of the calibration curve,
* increasing the used surface area (S) or
* decreasing the added volume of enzyme solution (M_E and also V_v).

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[1] G.W. Snedecor, W.G. Codran: Statistical methods, 1967.

[2] SAS/STAT User's Guide, Release 6.03, Procedure GLM.

CHAPTER 3

SAVINASE™ ADSORPTION ON POLYSTYRENE LATICES AND GLASS

Abstract

In this chapter the adsorption mechanism of the alkaline protease Savinase™ from *Bacillus lentus* is investigated. The interactions of importance for the adsorption of this protease at solid-liquid interfaces were examined by alteration of the experimental conditions such as pH, ionic strength, temperature and surface characteristics.

The adsorption of Savinase at solid-water interfaces is determined by electrostatic interactions between the surface and the enzyme, dehydration of hydrophobic interfaces and lateral interactions between the adsorbed Savinase molecules. The contribution of the dehydration of hydrophobic surfaces to the driving force is less than for the adsorption of proteins, such as RNase, reported in the literature. It was concluded that the enzyme adsorbs in the same spherical shape it has in solution and does not unfold upon adsorption.

As the adsorption depended strongly on the electrostatic interactions between the surface and the enzyme, it was concluded that under the experimental conditions examined here the contributions of electrostatic lateral repulsion and dehydration of hydrophobic parts of the surface to the free energy are of the same order of magnitude. The range of the lateral repulsion extended much further than the Debye length. The relative importance of the electrostatic interaction resulted in a decrease of adsorption on a negatively charged surface with increasing pH. Under attractive electrostatic conditions the adsorbed amount decreased with increasing ionic strength. The uptake of an extra Ca^{2+} ion in the weak calcium binding site of Savinase increases the adsorption at a negatively charged interface, probably because of the increased positive charge of the enzyme. The weak binding site proved to be selective for calcium ions as magnesium ions were not sequestered.

The adsorption proved to be dynamic (protein molecules replacing previously adsorbed ones) although it was not reversible against dilution. The adsorption on a hydrophobic surface was reversible towards changes in pH and ionic strength. The adsorption of inhibited Savinase was temperature independent in contrast to that of active Savinase at 30°C which was strongly determined by enzymatic autolysis.

3.1 Introduction

In this chapter the adsorption mechanism of the alkaline protease Savinase™ from *Bacillus lentus* is investigated. The interactions of importance for the adsorption of this protease at solid-liquid interfaces were examined by alteration of the experimental conditions such as pH, ionic strength, temperature and surface characteristics. The amount adsorbed was measured as a function of the concentration in solution after an incubation time that exceeded the time necessary to reach steady state. Two parts of the adsorption isotherm are considered. The slope of the initial, rising part of the isotherm (at low enzyme concentration) indicates the affinity of the enzyme for the surface. At higher enzyme concentrations the adsorbed amount reaches a plateau due to filling of the surface, which is affected by lateral repulsion between the adsorbed enzyme molecules. Conclusions about the interactions of importance for adsorption will be drawn from the properties of both parts of the isotherm under various experimental conditions.

In the main part of this study we used Savinase that was inactivated by PMSF (PMS-Savinase) as a protease is capable of hydrolysing itself. In order to use PMS-Savinase as a model for active Savinase both enzymes have to have similar adsorption characteristics. The results of the comparison between PMSF-inhibited and active Savinase will also be reported. The experimental techniques necessary to obtain reliable results were discussed in Chapter 2 and the characteristics of Savinase together with an overview over the literature on protein adsorption were given in Chapter 1.

3.2 Experimental

3.2.1 Materials

Enzyme

Savinase was isolated from a commercial preparation (ex NOVO-Nordisk, Denmark) by the Bio- and Nutrition Sciences Department, URL Vlaardingen. The preparation method for PMSF-inhibited Savinase was described in Appendix 2.1. The physical properties of Savinase and PMS-Savinase are summarized in Table 3.1.

Table 3.1 Physical properties of Savinase and PMS-Savinase.

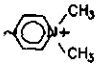
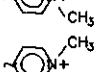
Molecular weight	27000 g mol^{-1}
Isoelectric point	pH = 10
Residual activity of PMS-Savinase	< 0.1 %
Radius of the spherical molecule	2.3 nm

Latices

The emulsifier-free latices indicated with A, B and C were kindly supplied by Dr. W. Norde (Wageningen Agricultural University). The PS-latex D and the latex with carboxyl surface groups were supplied by the Interfacial Dynamics Corporation, Portland, U.S.A.. The negatively charged latices A and B were prepared according to the procedures published by Furusawa et al [1]. The positively charged latices were prepared following the procedure of Blaakmeer et al [2]. The surface charges were determined by potentiometric titration.

The surface area was calculated from the specific surface or Sauter diameters ($D_{3,2}$) [3] that were determined with a SympaTec Helos particle sizer. Properties of four latices used in this study are summarized in Table 3.2. The zeta-potentials of the latices are summarized in Table 3.4 and discussed in section 3.3.1. They were measured in the buffer solutions as used in the adsorption experiments with a Malvern Zeta-sizer 3, a Multi 8, 7032 CN correlator and version 2.12 application software.

Table 3.2 Some physical properties of PS-latices.

	Surface group	σ / (μC/cm^2)	D_{32} / (nm)
Latex A−−	OSO_3^-	−11.6	495
Latex B−	OSO_3^-	−4.2	483
Latex C+		7.8	676
Latex D++		15.9	495
Carboxyl PS	OCO_2^-	−2.9	984

Glass

As a hydrophilic negatively charged surface we used Ballotini lead glass beads (code 1214K01 of Tamson, Zoetermeer). The diameter of these beads was in the range of 1 to 53 μm. The density of the glass was 2.9 g cm^{-3} and the average surface area was 0.2419 m^2 g^{-1} as determined with a Sympatec Helos particle size analyzer.

The beads were cleaned by soaking in a concentrated HCl/HNO_3 (3:1 w/w) solution. Afterwards they were thoroughly rinsed with deionized water and dried until constant

weight at 110°C [4]. Before the experiment, the glass beads were rinsed at least three times with the buffer solution used in the experiment.

3.2.2 Buffer solutions

Sodium tetraborate, $Na_2B_4O_7.10H_2O$, normally known as Borax (ex Merck) was used in combination with HCl or NaOH as a buffer to bring both pH and ionic strength to the desired values. The final compositions of the buffer solutions are shown in Table 3.3. The borate ion did not influence the adsorption of PMS-Savinase as was concluded from adsorption measurement on latex A at pH=8 and 10, for solutions with low and high buffer concentrations at a constant ionic strength of 0.05 M.

The buffering capacity of Borax pH=11 solutions was low even in solutions with an ionic strength of 0.05 M. Therefore addition of the concentrated enzyme solutions and the PS-latex caused a decrease of pH by a maximum of 0.5 units. The ionic strengths of the solutions were calculated assuming that the borate ion is divalent. The possible formation of large complexes of the borate ions was not taken into account [5].

Table 3.3 Buffer compositions.

pH	Ionic strength (M)	Indication	[Borax] (mM)	[HCl] (mM)	[NaOH] (mM)	[NaCl] (mM)	[$CaCl_2 2H_2O$] (mM)
7	0.01		2.6	5.1			
8	0.01		2.8	4.4			
8	0.05	high [buffer]	13.3	19.6			
8	0.05	low [buffer]-Ca	2.8	4.4			13
8	0.05	low [buffer]-Na	2.8	4.4		39	
8	0.10		13.3	19.6		50	
10	0.01		2.8		4.2		
10	0.05	high [buffer]	13.7		17.5		
10	0.05	low [buffer]-Ca	2.8		4.7		13
10	0.05	low [buffer]-Na	2.8		4.7	39	
10	0.10		13.7		17.5	50	
11	0.05		12.5		25.3		
11	0.10	low [buffer]	12.5		25.5	50	

The LiF- and $CsClO_4$-containing solutions consisted of Borax compositions identical to the "low [buffer]-Na" buffers. The LiF and $CsClO_4$ concentrations were identical to the NaCl concentration. The $MgCl_2$- and $CaCl_2$-containing solutions were prepared similarly.

3.2.3 Protein concentration determination

The principle and the experimental procedure for the Lowry colouring method and the measurement of the proteolytic activity on AAPF were described in Chapter 2. The calcium, magnesium and cesium containing solutions needed an extra filtration step with an Acrodisc 0.2 μm filter (ex Gelman Sciences) to remove the insoluble carbonate and hydroxide salts after the second colouring step in the Lowry protein determination. This filtration step did not influence the detected concentration.

3.3 Results

The adsorption values are measured at a temperature of 3 °C and after 60 minutes (PMS-Savinase) or 30 minutes (Savinase) contact time unless mentioned otherwise. These contact times are short when compared with most protein adsorption studies [6,7,8,9, 10,11] but not exceptional [4,12]. However, PMS-Savinase adsorption proved to reach the steady state (equilibrium) even within 15 minutes (see Chapter 2). Contact times longer than 1 hour had to be avoided because of autolysis, even for the inhibited protease. The adsorption values at an equilibrium concentration of 0.1 mg ml^{-1} are taken as plateau values.

3.3.1 Zeta-potentials of PS-latices

The ζ-potentials of the three latices, used in this study, were measured in buffer solutions identical to those used for the adsorption experiments (Table 3.4). The ζ-potential decreased with increasing ionic strength as expected. The differences between the potentials in solutions with "low" and "high" Borax concentrations, and the pH dependence of this difference, were probably caused by differences in ionic strength due to small inaccuracies in the calculation of the borate ion contribution (section 3.2.2). The ζ-potentials of the negatively charged latices A and B did not vary significantly with pH.

The fact that the ζ-potentials of latex B (surface charge $= -4.2$ $\mu C\,cm^{-2}$) were higher than those of latex A ($\sigma = -11.6$ $\mu C\,cm^{-2}$) was also observed by Elgersma [13] but cannot (yet) be explained by double-layer theories. The much lower ζ-potential of both negatively charged latices in presence of Ca^{2+} ions is an indication of specific adsorption of calcium ions on the latex particles.

The latices with a positive surface charge turned out to have negative ζ-potentials at all pH values. Charge reversal of positively charged latices is reported to be caused by adsorption of silicates from the glass wall of the zeta-sizer cell on the amidine latex [2].

Table 3.4 Zeta-potentials of PS-latices in buffer solutions.

pH	Ionic strength (M)	zeta–potential Latex A– –	Latex B–	Latex C+	Indication
6	0.01	−67.1			
7	0.01	−65.1			
8	0.01	−39.4	−76.5	−33.4	
8	0.05	−46.4	−51.3	−25.2	high [buffer]
8	0.05	−44.3	−46.6	−17.4	low [buffer]–Na
8	0.05	−13.1	−12.2		low [buffer]–Ca
8	0.10	−34.0			
10	0.01	−71.3	−78.0		
10	0.05	−53.6	−51.6		high [buffer]
10	0.05	−45.7	−44.2		low [buffer]–Na
10	0.05	−19.7	−20.8		low [buffer]–Ca
10	0.05	−20.9			low [buffer]–Mg
10	0.10	−40.1	−41.9		
11	0.05	−24.8	−43.8	−28.1	
11	0.10	−31.6	−32.2	−27.0	low [buffer]

The fact that the ζ-potential is influenced so strongly by only a small amount of negatively charged contaminant is probably caused by the low latex concentration that is used in the Zeta-sizer. The negative potential was not caused by specific adsorption of negatively charged borate ions because the ζ-potential of positively charged latices C and D in solutions containing only NaOH (with pH values ranging from 8 to 11) proved to be as negative as those in borax buffer solutions. In order to determine the actual ζ-potential of the positively charged latices it would be necessary to perform streaming potential measurements, as higher latex concentrations reduce the influence of contaminants on the measured potential.

3.3.2 Comparison of the adsorption of active and PMSF-inhibited Savinase

In order to prevent autolysis during the adsorption experiment, PMSF-inhibited Savinase was used in most experiments. The experiments reported in this section confirmed that the inhibition of Savinase with PMSF did not alter the enzyme's adsorption characteristics. In Figures 3.1 to 3.3 the adsorption of both PMS-Savinase and active Savinase are shown under three conditions. The adsorption values of active Savinase are averages of three contact times of 15, 30 and 60 minutes as no significant differences were detected. The adsorption of active and PMSF-inhibited Savinase did not differ

significantly although the relative deviation became somewhat larger with increasing pH. These small differences cannot be explained by autolysis during the adsorption experiment since the absence of any time dependence shows that even if some autolysis did occur it did not result in a decrease in adsorption.

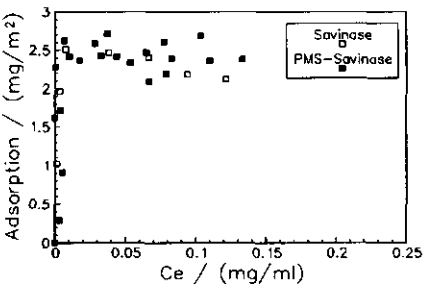


Figure 3.1 Adsorption isotherms of PMS-Savinase and Savinase on PS-latex A. Conditions: pH=8, I=0.01 M.

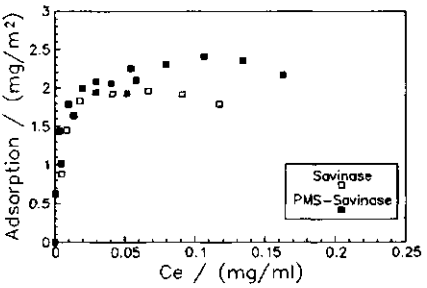


Figure 3.2 Adsorption isotherms of PMS-Savinase and Savinase on PS-latex A. Conditions: pH=10, I=0.01 M.

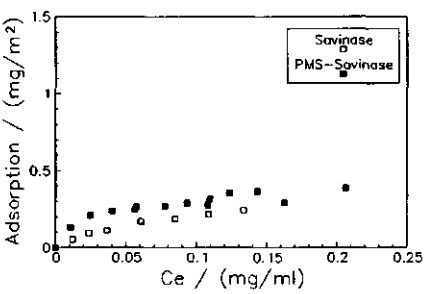


Figure 3.3 Adsorption isotherms of PMS-Savinase and Savinase on PS-latex A. Conditions: pH=11, I=0.05 M.

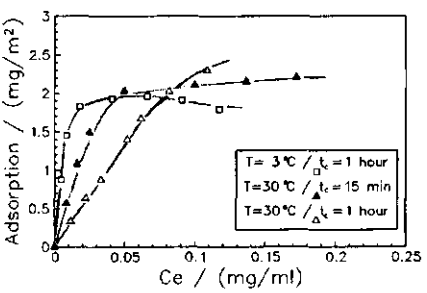


Figure 3.4 Temperature and time dependence of Savinase adsorption on PS-latex A. Conditions: pH=10, I=0.01 M.

At higher temperatures autolysis does play an important role in the determination of the adsorption isotherm of *active* Savinase. In Figure 3.4 the adsorption of active Savinase is shown as a function of enzyme concentration after 15 minutes and 60 minutes contact time. The strong time dependence of the adsorption at 30°C is absent at 3°C (t_c=60 minutes is shown in Figure 3.4). As the intrinsic enzyme adsorption was observed to be temperature independent (section 3.3.7) we concluded that autolysis of the protease influenced adsorption strongly. The shape of the isotherm at longer contact times can be explained by the low affinity of the autolysed fragments for the surface as concluded in Chapter 2. At low concentrations the surface was not completely covered as there was not enough undegraded enzyme to saturate it. The autolysed fragments remained in solution

keeping the protein concentration high. However, at higher equilibrium concentrations the amount of undegraded enzyme was enough to occupy the surface resulting in a seemingly normal adsorbed amount. The increase of adsorption at even higher concentrations could be explained by the adsorption of complexes of undegraded Savinase with one or more fragments, so-called product inhibited Savinase.

These results showed that PMS-Savinase is a good model for active Savinase and it gives us the opportunity to determine the intrinsic adsorption in the absence of autolysis, even at higher temperatures.

3.3.3 Savinase adsorption on various surfaces

Figures 3.5 and 3.6 show that the adsorption of positively charged enzyme (at pH=8) on negatively charged latices (A and B) was of the order of a monolayer coverage. However, PMS-Savinase hardly adsorbed at all on positively charged latices under these conditions. This indicates that electrostatic interactions between the enzyme and the surface are an important driving force for PMS-Savinase adsorption.

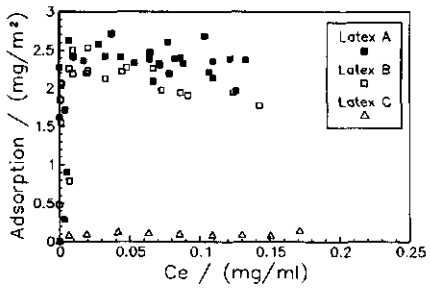


Figure 3.5 Adsorption isotherms of PMS-Savinase on various PS-latices. Conditions: pH=8, I=0.01 M.

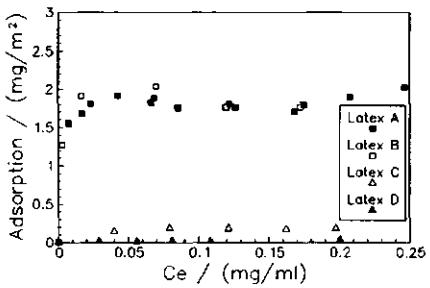


Figure 3.6 Adsorption isotherms of PMS-Savinase on various PS-latices. Conditions: pH=8, I=0.05 M.

Apart from this general conclusion, some of the details shown in Figures 3.5 to 3.9 are not that easily interpreted. At pH=8 and an ionic strength of 0.05 M the adsorption on latex A was identical to that on latex B (Figure 3.6) just as was the case at 0.01 M (Figure 3.5). One would have expected the higher surface charge of latex A to result in a higher adsorption under high ionic strength conditions. However, Figure 3.8 shows that at pH=10 and I=0.05 M the adsorption was *lower* on latex A than on latex B (the decrease in adsorption on latex B in Figure 3.7 will be discussed in 3.4.1.3). This could indicate that under these conditions dehydration of the hydrophobic polystyrene is the more important at this pH. The higher the surface charge the smaller the hydrophobic patches and the more hydrophilic surface groups will be dehydrated by protein adsorption

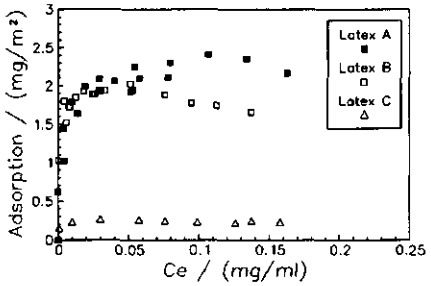


Figure 3.7 Adsorption isotherms of PMS-Savinase on various PS-latices. Conditions: pH=10, I=0.01 M.

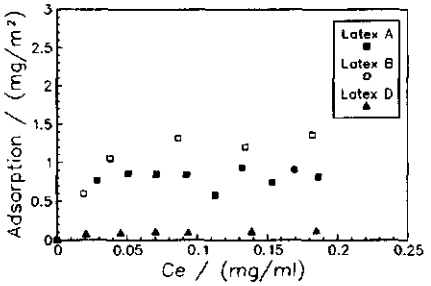


Figure 3.8 Adsorption isotherms of PMS-Savinase on various PS-latices. Conditions: pH=10, I=0.05 M.

(see also 3.3). This could result in a lower adsorption on latex A. The adsorption isotherms at pH=10 (Figures 3.7 and 3.8) are very similar to those measured at pH=8. At pH=10 the protein has no net charge but the adsorption shows that there was an effective attractive interaction between the enzyme and the negatively charged latices. Low adsorption on the positively charged one, together with an ionic strength dependence indicates that there is an attractive electrostatic interaction. It is not yet clear if these results could be explained by an asymmetrical distribution of charges on the enzyme, so-called "mosaic charges", to which we shall return in Chapter 4.

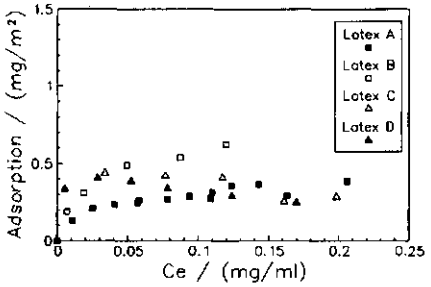


Figure 3.9 Adsorption isotherms of PMS-Savinase on various PS-latices. Conditions: pH=11, I=0.05 M.

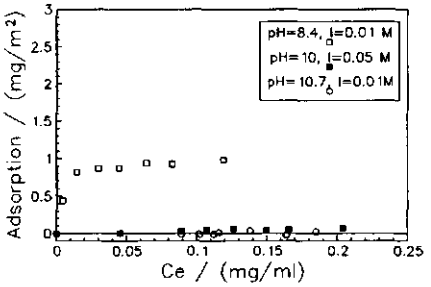


Figure 3.10 PMS-Savinase isotherms on glass at various pH values.

As latex A and B consisted of sulphate-groups attached to polystyrene while latex C and D did not, we verified that no specific interaction occurred between the sulphate group and the enzyme. The adsorption isotherms on PS-latex with carboxyl and sulphate surface groups at pH=8 and an ionic strength of 0.01 M proved to be identical. Hence, the observed difference between positively and negatively charged latices can not be attributed to specific interactions between the enzyme and the sulphate group.

In Figure 3.10 the adsorption of PMS-Savinase on glass is shown under various pH and ionic strength conditions. These experiments showed clearly that adsorption on a hydrophilic surface occurs only when the electrostatic interaction is attractive. It can be concluded that dehydration of hydrophobic patches on the enzyme and Van der Waals interactions between the enzyme and the glass together are not enough to overcome the lateral repulsions between the enzymes even at pH=10 (the isoelectric point of Savinase).

The fact that the adsorption onto hydrophobic surfaces is under all conditions larger than that on hydrophilic glass indicated that dehydration of the hydrophobic interfaces is an important additional driving force for Savinase adsorption.

3.3.4 Variation of pH

Figure 3.11 shows the plateau values as a function of pH. The adsorption of PMS-Savinase on negatively charged latices decreases with increasing pH. This indicates again that electrostatic interaction is an important driving force for the adsorption. The fact that there is still enzyme adsorbed at pH=11 showed again that Van der Waals and hydrophobic interactions partially determine the adsorption of PMS-Savinase.

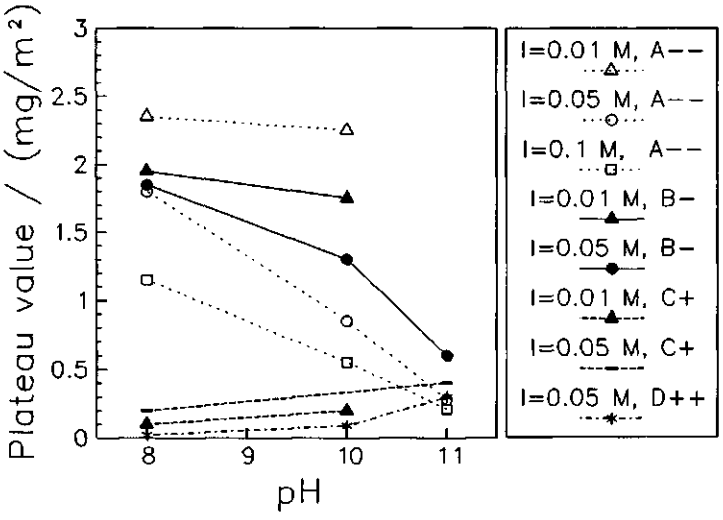


Figure 3.11 pH-dependence of plateau values of PMS-Savinase on various PS-latices at C_e=0.1 mg/ml.

The plateau values at pH=6 and 7 (I=0.01 M) are not shown in Figure 3.11. These values were identical to the pH=8 plateau value namely 2.4 mg m^{-2}. Although no maximum in plateau value was observed, these data are not conclusive about the existence

of such a maximum around the isoelectric point of the protein-surface complex similar to that measured for HPA and other "unfolding" proteins [13]. As the ζ-potential of the particles that were covered with enzyme at pH=6 was negative, the complex is negative across the whole experimentally available pH range. Therefore it can be concluded that the isoelectric point of the enzyme-sorbent complex, and hence also the maximum in plateau value (if it exists) is below pH=6.

On a positively charged latex the adsorption *increased* with increasing pH; again as expected for electrostatically driven adsorption. The observation that the adsorbed amount at pH=11 was not as high as for example at pH=8 on a negatively charged latex, could be explained by the fact that the surface charges of the positively charged latex are titrated above pH=10 [2]. Alternatively, there could be a decrease in effective positive surface charge density by anion adsorption on the latex.

In addition to the variation in plateau value with pH we also examined the change in the slope of the rising part of the isotherm at low enzyme concentration, which is a measure of the affinity of the adsorption. The "high affinity" character of the isotherms made it necessary to use a highly sensitive concentration determination method. We therefore measured these isotherms with the AAPF method using active Savinase instead of PMS-Savinase. It was shown above (section 3.3.2) that the adsorption characteristics of both forms of Savinase are identical.

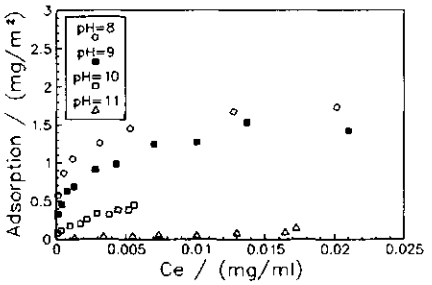


Figure 3.12 Initial parts of the adsorption isotherms of Savinase at various pH-values. Conditions: I=0.05 M, PS-latex A, AAPF-method.

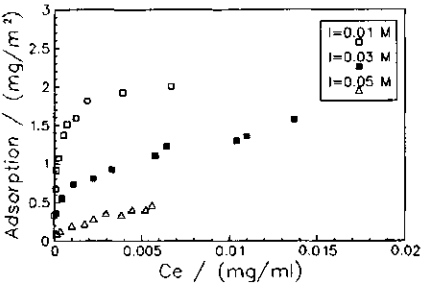


Figure 3.13 Initial parts of the adsorption isotherms of Savinase at various ionic strengths. Conditions: pH=10, PS-latex A, AAPF method.

In Figure 3.12 the pH dependence of the low concentration part of the isotherms of active Savinase on negatively charged PS-latex is shown. It is clear that the above reported variation in plateau value is reflected in the affinity of the isotherms. Under attractive electrostatic conditions the isotherms are high affinity (an infinite slope) while at higher pH values the slope decreases just as the plateau value does. This indicates that the

plateau value is determined by the balance of enzyme-interface interaction and enzyme-enzyme repulsion and not so much by the conformation of the enzyme at the interface. In this context it is good to note that the isotherms did not cross at low concentrations; this also supports the conclusion that the enzyme does not unfold upon adsorption as will be discussed in section 3.4.1.

3.3.5 Ionic strength dependence of PMS-Savinase adsorption

Figure 3.14 shows the resulting plateau values as a function of ionic strength. Under electrostatically attractive conditions the adsorption decreases with increasing ionic strength. This would be expected for electrostatically driven adsorption as increased shielding of the charges by the salt ions will decrease the attractive interaction. The only exception is the adsorption at pH=8 on latex B that was not significantly different at I=0.01 and 0.05 M. Just as it was the case for the pH dependence, the change in plateau value with ionic strength reflected the variation of the affinity of the isotherms at low concentrations. In Figure 3.13 the adsorption isotherms of Savinase on PS-latex A are shown as measured with the AAPF concentration determination method.

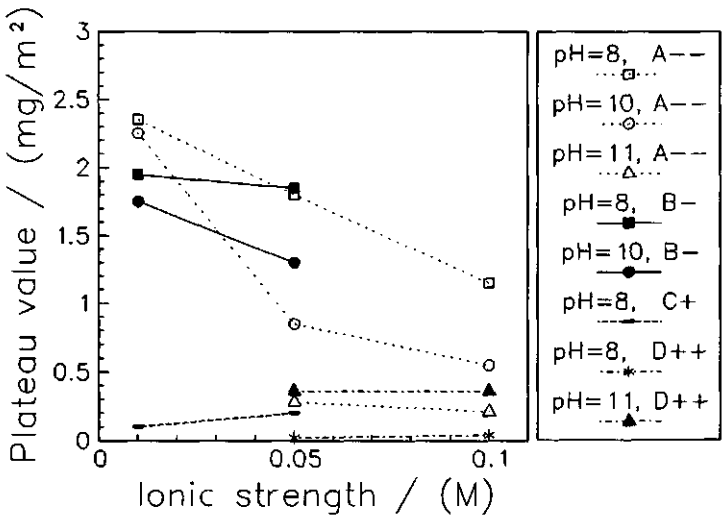


Figure 3.14 Ionic strength dependence of plateau values of PMS-Savinase on various PS-latices with C_e=0.1 mg/ml.

At pH=10 a strong ionic strength dependence was observed that was unexpected for the adsorption of a protein without net charge at its isoelectric point. This could indicate that not only the net charge of the enzyme but also that the charge distribution on the enzyme molecule determines the interaction with the charged interface.

The ionic strength dependence was also investigated for two electrostatically repulsive conditions. Normally an increase in ionic strength would lead to an increased adsorption due to a decrease in electrostatic repulsion between both the enzyme and the surface and the adsorbed enzymes themselves (lateral repulsion). As the electrostatic repulsion is strong enough to restrict the plateau value of the isotherms to less than 20% of the maximum value it would be expected that increasing the ionic strength would lead to a significant increase in plateau value. However, as shown in Figure 3.14 increasing the ionic strength at pH=8 led only to a small increase in adsorption on latex C while the adsorption at pH=11 on latex A was independent of ionic strength. These results correspond with ones obtained with the Savinase variants that will be reported in Chapter 4 (section 4.3.3). At the moment we cannot explain these results.

3.3.6 The influence of the type of salt ions on PMS-Savinase adsorption

Ca^{2+} and Mg^{2+} ions

Ca^{2+} is an important ion in the application of Savinase in detergent formulations. Calcium is known to be sequestered by Savinase in two so-called calcium binding sites. One calcium is strongly bound in an approximately octahedral coordination sphere [14]. This binding site is thought to be occupied with calcium under normal conditions. The binding of the second calcium ion is not as strong due to a less effective coordination. The calcium-ions in subtilisins inhibit their thermal denaturation and/or the autolysis rate of the proteases [15,16,17,18] by an as yet unknown mechanism. It is known that the calcium binding sites of subtilisins, such as subtilisin BPN', are selective for Ca^{2+} and do not bind other divalent cations such as Mg^{2+} [18]. This selectivity was observed for several proteins with differences in binding affinities for Ca^{2+} and Mg^{2+} of up to 10,000-fold. This has been explained by Stuart et al. in terms of the rigidity of the area of Ca^{2+} binding in α-lactalbumin where the protein is apparently unable to fold around the smaller Mg^{2+} ion [19]. In addition to increasing the stability, binding of calcium to the enzyme will also increase the positive charge and hence its isoelectric point. Therefore addition of Ca^{2+} is expected to lead to an increased adsorption at a negatively charged interface.

Association with the enzyme is not the only way in which calcium could influence the adsorption. Calcium ions interact with PS-latex A at both pH=8 and at pH=10 as it could be concluded from the ζ-potential measurements (Table 3.4). In order to determine which of these two possible interactions, with the enzyme or with the negatively charged PS-latex, influenced the adsorption we compared the adsorption in the presence of calcium to that containing magnesium in solution. Magnesium also interacted with PS-latex A resulting in a similar ζ-potential but it is not expected to bind to Savinase through

the calcium binding sites.

In Figure 3.16 the adsorption of Savinase on PS-latex A at pH=10 is shown to increase by a factor of 2 upon replacement of most of the Na^+ by Ca^{2+} ions in solution keeping the ionic strength constant. However, comparable replacement of Na^+ by Mg^{2+} did not alter the adsorption at all. The fact that calcium increased the adsorption while magnesium did not, proved that the interaction of calcium with the Savinase caused the increase and that Savinase, just as subtilisin BPN', interacts selectively with calcium.

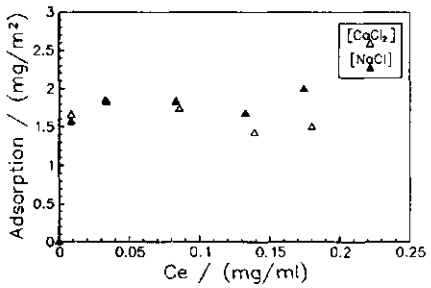


Figure 3.15 PMS-Savinase adsorption isotherms on PS-latex A with Ca^{2+} and Na^+. Conditions: pH=8, I=0.05 M.

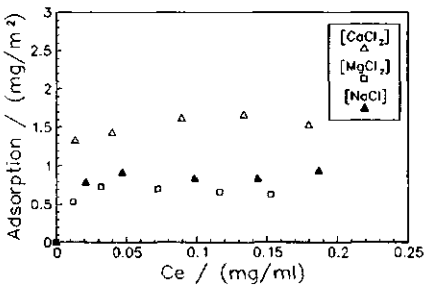


Figure 3.16 PMS-Savinase adsorption isotherms with Ca^{2+}, Mg^{2+} and Na^+ on PS-latex A. Conditions: pH=10, I=0.05 M.

The fact that the adsorption at pH=8 (Figure 3.15) was not influenced by the presence of calcium ions could indicate that the weak binding site sequestered the calcium ion better at pH=10 than at pH=8 (similar phenomena are reported for other proteins such as e.g. thermolysin [20]). Examination of the X-ray structure of Savinase reveals that there are two tyrosine residues, number 167 and 171, near the weak calcium binding site which (normally) have a pK value around pH=10. We could therefore tentatively explain a pH dependence of calcium binding by the titration of these tyrosine residues (which become negatively charged above pH=9). This could be verified by determination of the total charge of the PMS-Savinase molecule with Capillary Zone Electrophoresis in presence and absence of Ca^{2+} ions at both pH=8 and at pH=10.

Enzyme adsorption and the Hofmeister series

In addition to the above mentioned phenomena it was investigated whether there is a relation between the salting-out capability of a salt and its influence on enzyme adsorption using, in addition to the ions reported in the previous paragraph, three monovalent salts. They were chosen in such a way that a large anion was coupled to a large cation so that the order of anions and cations in the Hofmeister series was identical (the salting-out capacity of LiF > NaCl > $CsClO_4$ and that of NaCl > $MgCl_2$).

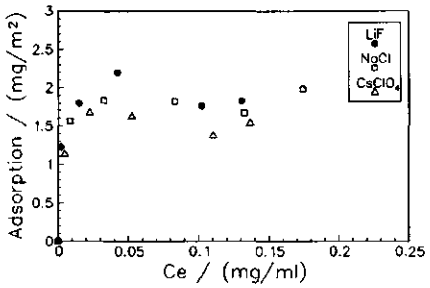


Figure 3.17 PMS-Savinase isotherms on PS-latex A in presence of LiF, NaCl and $CsClO_4$. Conditions: pH=8, I=0.05 M.

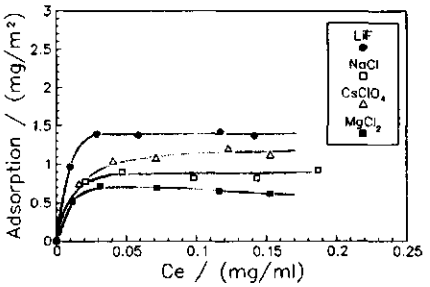


Figure 3.18 PMS-Savinase isotherms on PS-latex A in presence of LiF, NaCl, $CsClO_4$ and $MgCl_2$. Conditions: pH=10, I=0.05 M.

In the Figures 3.17, 3.18 and 3.19 the isotherms are shown in the presence of these salts at pH=8, 10 and 11, respectively. At pH=8 and 11 no significant influence of the presence of various salt ions could be observed. Only at pH=10 the adsorption depends on the nature of the ions present in solution. The dependency can not be explained by the Hofmeister series as the adsorption was higher with $CsClO_4$.

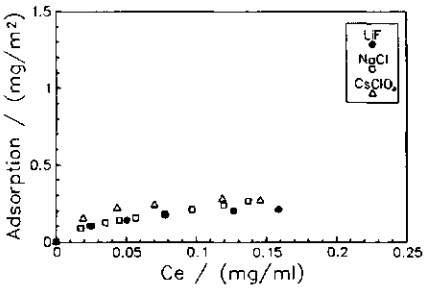


Figure 3.19 PMS-Savinase adsorption isotherms on PS-latex A in presences of LiF, NaCl and $CsClO_4$. Conditions: pH=11, I=0.05 M.

We can not yet explain the specific influence of salt ions on the enzyme adsorption just as their influence on enzyme kinetics [21] is yet unknown. Although the phenomenon is not yet understood, the fact that these "salt-effects" occurred only at pH=10 indicated that the change in free energy is only small and therefore it influenced enzyme adsorption only in the absence of other strong attractive or repulsive interactions.

3.3.7 Temperature dependence

In Figure 3.20 the adsorption isotherms of PMS-Savinase at 3°C and 30°C are shown at attractive and repulsive electrostatic conditions. The plateau values of the isotherms do not differ significantly at the two temperatures. The same conclusion can be drawn from Figure 3.21 for the adsorption at 3°, 15° and 30°C on latex A. The fact that all isotherms are very high affinity hampers us in determining the contribution of the enthalpy to free energy gain of the system upon adsorption under attractive electrostatic conditions.

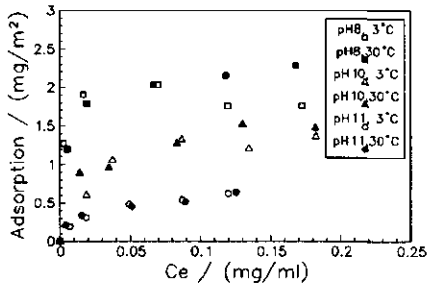


Figure 3.20 Temperature dependence of PMS-Savinase adsorption isotherms on PS-latex B with I=0.05 M.

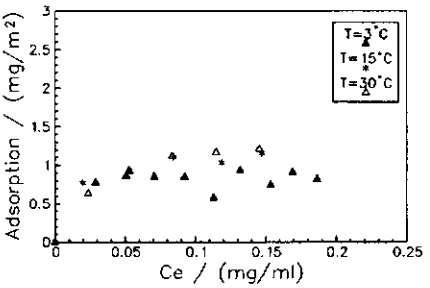


Figure 3.21 Temperature dependence of PMS-Savinase adsorption on PS-latex A at pH=10 and I=0.05 M.

Only under repulsive conditions (at pH=11) can it be seen that not only the plateau values but also the slopes of the isotherms are similar. According to Le Chatelier's rule it can be concluded that the enthalpic contribution to PMS-Savinase adsorption is very small under these conditions. The fact that the adsorption occurred spontaneously meant that an entropy change (e.g. dehydration of hydrophobic interfaces) was the driving force for PMS-Savinase adsorption at pH=11.

3.3.8 Reversibility of PMS-Savinase adsorption

The reversibility of the adsorption is often examined as a means to determine the way a protein is adsorbed at an interface. In the literature it is sometimes implicitly assumed that protein adsorption is completely reversible. However, for large molecules such as proteins, with many contact points at the surface and possible unfolding upon adsorption, it does not seem an obvious assumption as indeed was proved experimentally by Van der Scheer [22]. He showed that HPA adsorption increased if the experimental conditions were changed from low to high adsorption conditions. However, the reverse changes did not lead to desorption. We examined three types of reversibility of Savinase adsorption on latex A.

The first experiment was an "addition experiment", a stepwise increase of PMS-Savinase concentration, at two conditions namely pH=8, I=0.01 M and pH=10, I=0.05 M. In the first step we added enough enzyme to reach an adsorption of 0.58 and 0.34 mg m^{-2}, respectively. After 30 minutes contact time we added the second amount of enzyme, enough to reach the plateau value according to the isotherms reported above. The amounts adsorbed after another 30 minutes contact time were compared with experiments in which identical total quantities of enzyme were added at once. There appeared to be no difference between the adsorbed amounts after one and two step additions.

The reversed experiment is the determination of the desorption after dilution of the solution in contact with the surface at which the enzyme was adsorbed. If the adsorption is completely reversible the isotherm should be followed not only in the direction of increasing but also of decreasing equilibrium concentration. As the adsorption isotherms increased sharply at low concentrations under almost all experimental conditions, the reversibility of solvent substitution could only be verified experimentally at pH=9 and an ionic strength of 0.05 M using active enzyme and the AAPF detection method. In figure 3.22 four adsorption values detected before and after replacing 2/3 of the enzyme solution with buffer solution are shown together with the "normal" isotherm (see also Figure 3.12). These results show that, once adsorbed, the Savinase was not displaced by solvent molecules.

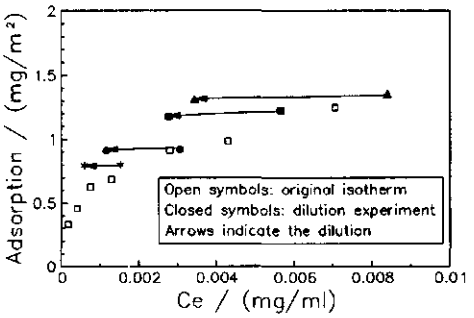


Figure 3.22 Irreversibility of Savinase adsorption against dilution. Conditions: pH=9, I=0.05 M, t_c=30 min, PS-latex A.

The second type of experiment consisted of changing other experimental conditions that should increase or decrease the adsorbed amount. After 30 minutes contact time we changed the pH from 8 to 11 and from 11 to 8 at I=0.05 and in experiments at both pH=8 and 10 the ionic strength was increased from 0.01 to 0.1 M. These experiments showed that the adsorption was completely reversible towards these changes in experimental conditions. The adsorption was in all cases identical to the measured isotherms under constant experimental conditions. The reversibility of Savinase adsorption against a change from high to low adsorption conditions is unlike that reported for HPA on polystyrene latices [22].

The last type of reversibility that was determined was that against replacement by protein molecules with identical adsorption characteristics. We exchanged adsorbed *active* Savinase for PMS-Savinase and used the enzymatic activity (measured with AAPF method) as label to detect this exchange. This experiment showed that quantitative exchange took place within 30 minutes and this proved that Savinase adsorption is indeed a dynamic equilibrium at pH=8, I=0.01 M and T=3°C. At the same time these results indicated that no significant loss of enzymatic activity was measured due to adsorption.

3.4 Discussion

3.4.1 The shape of the adsorption isotherm

Monolayer coverage

The maximum amount that can be adsorbed on a surface can be calculated when the size and geometry of the molecule are known. The structure of Savinase is known from X-ray analysis and we will assume that the molecular structure in solution is identical to the one in the crystal. The Savinase molecule appears to be almost spherical. The cylinder with the smallest base that can envelope the "backbone" of the protein molecule was calculated to have a radius of the base of 2.27 nm and the length of this cylinder is 4.6 nm. A close-packed surface could then consist of about 93 nmol m^{-2} or 2.5 mg m^{-2}. Under strongly attractive electrostatic conditions the experimentally determined plateau value corresponded to this maximum. This is a clear indication that the conformation of the enzyme on the surface is comparable with the one in solution.

The decrease of adsorption with increasing enzyme concentrations

Sometimes we observed that the plateau value of the adsorption isotherm decreased slightly when the equilibrium concentration was increased (e.g. Figures 3.7 and 3.28). Extension of the concentration range to higher values showed that this decrease was significant and continued upon further increase of the concentration (Figure 3.23). Two explanations can be suggested: autolysis caused by small residual proteolytic activity in the PMS-Savinase samples and/or competition between the enzyme and a non-protein contamination of the system.

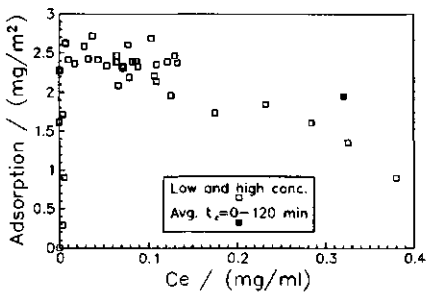


Figure 3.23 High concentration adsorption isotherm of PMS-Savinase on PS-latex A. Conditions: pH=8, I=0.01 M, t_c=60 min.

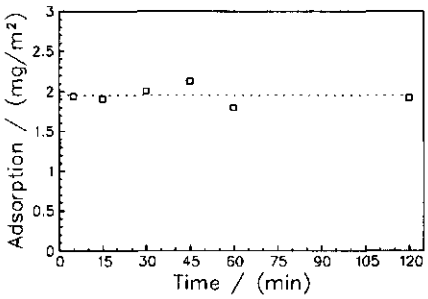


Figure 3.24 Time dependence of PMS-Savinase adsorption on PS-latex A at (high) C_e=0.32 mg/ml. Conditions: pH=8, I=0.01 M.

The first possible explanation was checked experimentally by measurement of the time dependence of the adsorption at high C_e. Figure 3.24 shows that autolysis could not cause

a decrease in adsorbed amount at low temperature and high initial concentrations. We therefore explain this phenomenon as caused by the presence of a small amount of one or more surface active (non-protein) components in the PMS-Savinase samples. These compounds adsorbed preferentially over the enzyme after the plateau value was reached (see also Chapter 2) thereby decreasing the adsorption with increasing protein and thus contaminant concentration. The fact that the decrease differed in magnitude when the adsorption was measured using various batches of PMS-Savinase (Figure 3.23) is in accordance with this interpretation. However, the origin of the contaminating component(s) is not known.

Variation of plateau value with experimental conditions

Changing the experimental conditions towards diminished electrostatic attraction one would expect a decrease in affinity of the isotherm but a similar plateau value. As long as the adsorption occurs spontaneously (i.e. the Gibbs free energy of the system decreases) one expects the plateau value to correspond to a completely covered surface. However, the comparison of the above reported experimentally determined isotherms showed that not only the affinity but also the plateau values of most of the PMS-Savinase isotherms varied with the experimental conditions (e.g. Figures 3.25 and 3.26). Only at low ionic strength did the plateau values on latex A remain constant between pH=8 and 10 (Figures 3.27 and 3.28).

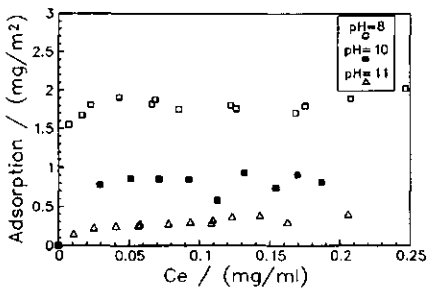


Figure 3.25 Adsorption isotherms of PMS-Savinase on PS-latex A at various pH values with I=0.05 M.

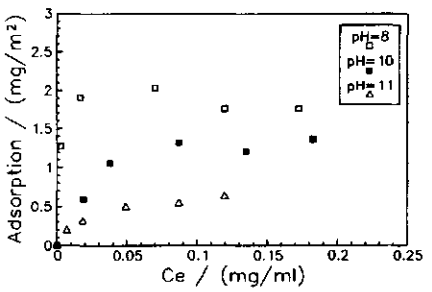


Figure 3.26 Adsorption isotherms of PMS-Savinase on PS-latex B at various pH values with I=0.05 M.

In principle the variation in plateau value could be explained in two different ways: either by unfolding of the enzyme or by lateral repulsion between the adsorbed enzymes. Both explanations will be discussed in the following section.

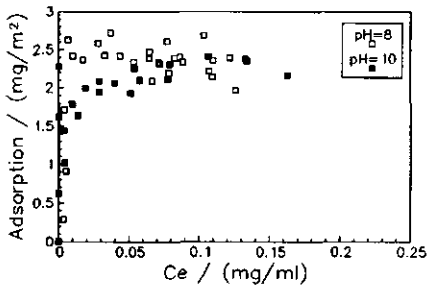


Figure 3.27 Adsorption isotherms of PMS-Savinase on PS-latex A at various pH values with I=0.01 M.

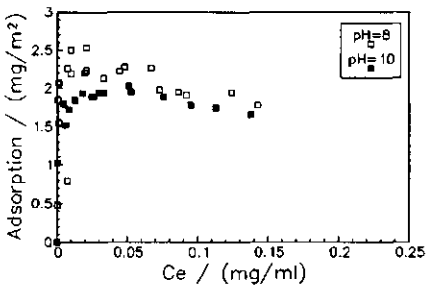


Figure 3.28 Adsorption isotherms of PMS-Savinase on PS-latex B at various pH values with I=0.01 M.

3.4.2 The adsorption mechanism

Unfolding at the interface

Our interpretation of the above reported complete set of experiments is that Savinase does not unfold upon adsorption on hydrophobic surfaces. This conclusion is based on the following arguments. Both "hard" and "soft" proteins, examined in the literature, adsorb on all hydrophobic surfaces irrespective of the electrostatic charges on the surfaces. The reason for this is clearly that the decrease in free energy by dehydration of the hydrophobic interface ($\Delta G = -15.7$ mJ m^{-2} at 3°C, [23]) together with the entropy gain by unfolding of the protein (release of 50 amino acids from a α-helix or β-sheet into a random coil corresponds to about $\Delta G = -70$ kT, [23]) could easily overcome any repulsive interactions. This is clearly not the case with PMS-Savinase as it does not adsorb on hydrophobic, positively charged, latices at pH values below the isoelectric point. As electrostatic interactions are of the same order for e.g. RNase and PMS-Savinase and significantly smaller than the contribution from dehydration and unfolding, we conclude that PMS-Savinase does not unfold upon adsorption. One should realize that in the case that a protein adsorbs as a sphere it not only keeps a low entropy conformation but that the dehydrated area at the interface is small and may decrease the entropy by structuring of the water between the spherical protein and the latex wall (Figure 3.29).

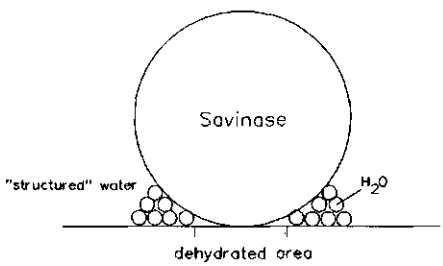


Figure 3.29 Schematic representation of Savinase adsorption.

Another reason for concluding that Savinase does not unfold is the fact that the plateau value corresponds to a molecular size corresponding to the compact X-ray structure. The plateau value at pH=6 is still 2.4 mg/m^2 even though the enzyme is strongly positively charged and the *intra*molecular electrostatic repulsion would be expected to favour unfolding.

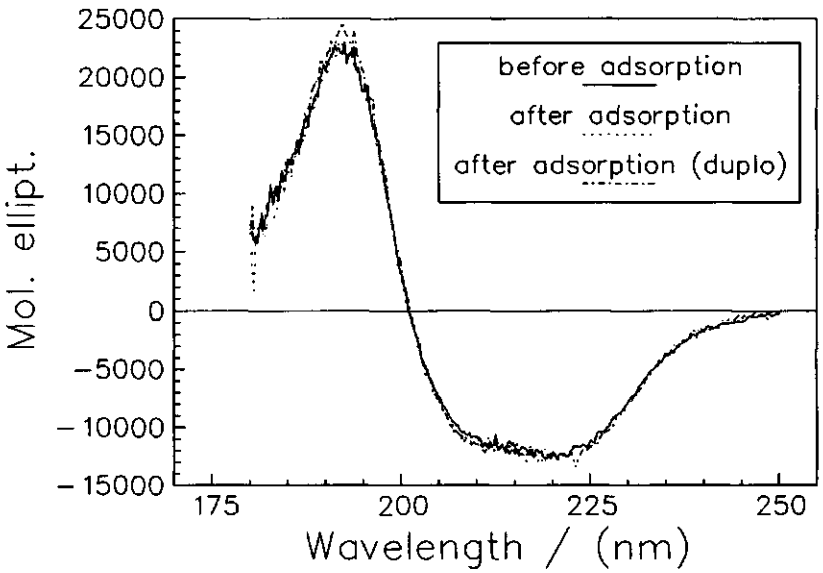


Figure 3.30 CD spectra of PMS-Savinase before and after adsorption. Conditions: C_0=0.85 mg/ml and C_e=0.69 mg/ml, pH=8, I=0.01 M.

The fact that the isotherms measured under various experimental conditions do not cross at low concentration is yet another indication. Under condition where the enzyme unfolds the plateau values of the isotherms is low as the number of molecules that "fit" on the surface is small. It also causes the isotherms to have steep initial slopes as the strong unfolding increases the affinity of the enzyme for the surface.

The absence of *irreversible* unfolding was checked by comparing the protein structure before and after it was brought in contact with PS-latex A for 2 hours at pH=8, I=0.01 M and T=3°C. The protein structure was determined from CD-spectra were measured with a Jobin Yvon Dichrograph VI CD spectroscope. Comparison of the CD-spectra (Figure 3.30) showed that no structural differences were induced by frequent adsorption and desorption (section 3.3.8).

Lateral repulsion between adsorbed enzymes

What causes the measured decrease in plateau value if (substantial) unfolding does not take place? In order to answer this question we have to realize that the only interaction opposing completion of a monolayer coverage is the lateral repulsion between the adsorbed enzymes. As dehydration of the interface and structural changes in the protein do not lead to a large decrease of free energy the adsorption isotherm will reach a complete monolayer only in the case of electrostatic attraction. However, when the electrostatic attraction decreases, the lateral repulsion "tips" the balance and results in only partial coverage of the surface.

According to the DLVO-theory the long-range character of the electrostatic lateral repulsion would be effective only within a distance of the order of a Debye length. However, the results shown above, indicate that the range of the lateral repulsion is much broader. However, when we take for example the maximum adsorption of 0.55 mg/m^2 at pH=10 and an ionic strength of 0.1 M (Figure 3.11) it is easily calculated that only 20% of the surface is covered with PMS-Savinase under these conditions. This corresponds to an intermolecular distance of about 10 nm while the Debye length (κ^{-1}) at 0.1 M ionic strength is only 1 nm. The amazing fact that lateral, repulsive, protein-protein interactions have a much longer range than predicted by DLVO-theory was observed (but not explained) before by other experimentors when measuring the forces between protein covered surfaces [24,25,26].

Using the data on adsorption at very low surface coverage we estimated the amount of Gibbs energy corresponding to the repulsive lateral interaction. In Figure 3.12 the adsorption on PS-latex A at pH=10 and an ionic strength of 0.05 M is shown to increase linearly within the concentration range shown. Therefore we assume that at these low surface coverages the lateral repulsion is absent. A rough estimation of the free energy of adsorption can be obtained using the simple Langmuir equation [27]:

$$\theta / (1-\theta) = C_e/55.5\ e^{-\Delta G/RT} \qquad (1)$$

where θ is the fractional surface coverage (adsorbed amount divided by the maximum adsorbed amount that can be accommodated on the surface i.e. in our case 2.5 mg/m^2),

C_e is the concentration in solution in mol l^{-1},

$\Delta G°$ is the change in Gibbs energy upon adsorption and

R is the gas constant and T the absolute temperature.

However, it must be remembered that Savinase adsorption is not reversible against dilution and that therefore one of the conditions for use of the Langmuir equation is not

met. We will use equation (1) only to approximate the order of the change in Gibbs energy in spite of this fact.

Using equation (1) with the adsorption values at pH=10 (Figure 3.12) the change in free energy upon adsorption is about -18 RT (-45 kJ mol^{-1}) in the absence of lateral repulsion. Before continuing with this value, we verified that it is of correct order of magnitude. Under the experimental conditions chosen here, it was previously concluded that the adsorption on PS-latex A was mainly driven by dehydration of the hydrophobic interface since the adsorption on negatively charged glass was negligible at pH=10 and at an ionic strength of 0.05 M (Figure 3.10). How large would the dehydrated surface area per molecule have to be to give a $\Delta G°=-18$ kT (where k is the Boltzmann constant). Dehydration of PS-latex gives a $\Delta G°$=-15.7 mJ m^{-2} [23] and thus 18 kT corresponds to 4.4 nm^2 dehydrated surface area per Savinase molecule. This is 25% of the projection area of a spherical molecule with a radius of 2.3 nm and this order of magnitude is in accordance with the conclusions drawn in the previous section that were summarized in Figure 3.29. We therefore conclude that the estimated order of magnitude of the change in Gibbs energy is correct.

As an adsorption isotherm reaches its plateau value the change in free energy for an extra enzyme molecule to adsorb must be zero. This would mean that at that surface coverage the lateral repulsion must be of the same order as the change in Gibbs energy upon adsorption at low surface coverage. Therefore the lateral repulsion is estimated to be also of the order of 45 kJ mol^{-1}. For net uncharged molecules at distances of 7.6 nm apart (calculated from the plateau value under these conditions, Figure 3.11) this is a very considerable repulsion which is unlikely to be caused by electrostatic interactions.

Comparing the Savinase adsorption mechanism to that of other proteins we conclude that the main difference is that Savinase adsorbs as a sphere and does not unfold significantly upon adsorption. This results in a relatively small attractive interaction due to the small area at the hydrophobic interface that is dehydrated. This brings about a strong dependence on electrostatic and (long range) lateral interactions. In Chapter 5 we will show that Savinase is not unique in this respect as Lipolase will be shown to adsorb in a similar way. Furthermore, limited data reported on the adsorption of subtilisin BPN' [28], another alkaline protease, indicated that this enzyme also adsorbed without unfolding and that the plateau value of the adsorption isotherm corresponded to a far from completely covered surface. This indicates that for BPN', just as it was the case for Savinase, the plateau value of the isotherm is determined by long range lateral repulsions instead of being determined by the degree of unfolding of the protein as it is the case for most blood proteins.

3.5 Conclusions

The adsorption of Savinase at solid-water interfaces is mainly determined by electrostatic interactions between the surface and the enzyme and to a lesser extent by dehydration of the hydrophobic interfaces and lateral interactions between the adsorbed Savinase molecules. The contribution of the dehydration of hydrophobic surfaces to the driving force was small compared to protein adsorption examined in the literature. It was concluded that the enzyme adsorbed as a sphere and did not unfold upon adsorption.

As the actual adsorption depends strongly on the electrostatic interactions between the surface and the enzyme, under the experimental conditions examined here, it was concluded that the contributions of electrostatic lateral repulsion and the dehydration of hydrophobic surface to the free energy of adsorption are of the same order of magnitude. The range of the lateral repulsion extended much further than the Debye length. The relative importance of the electrostatic interaction resulted in a decrease of adsorption on a negatively charged surface with an increase in pH. Under attractive electrostatic conditions the adsorbed amount decreased with increasing ionic strength. The uptake of an extra Ca^{2+} ion in the weak calcium binding site of Savinase increases the adsorption at a negatively charged interface probably because of the increased positive charge on the enzyme. This effect did not occur with the divalent magnesium ion.

The adsorption proved to be a dynamic equilibrium (protein molecules replacing previously adsorbed ones) although it was not reversible against dilution. The adsorption on a hydrophobic surface proved to be reversible towards changes in pH and ionic strength. The adsorption of inhibited Savinase was temperature independent while the adsorption of active Savinase at 30°C was strongly determined by enzymatic autolysis.

3.6 References

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CHAPTER 4

THE ADSORPTION OF SAVINASE VARIANTS ON POLYSTYRENE LATICES AND GLASS

Abstract

The adsorption of a set of closely related Savinase variants, made by protein engineering, was examined on several adsorbents under various experimental conditions. The six variants used in this study differed from "wild type" Savinase mainly in their electrostatic properties. Two parameters were altered as independently as possible: the isoelectric point and the number of charged residues.

On negatively charged PS-latex differences in electrostatic interaction dominated the differences between the adsorption of the various Savinase variants. The electrostatic characteristics of the variants could not be described completely on the basis of either the net charge of the protein as calculated with Tanford's equation or the mean surface potential. Altering the primary structure of the protein brought about changes in electrostatic properties which could not be explained properly without specifically considering the spatial distribution of the charged residues. Hydration of the enzyme was less important for the adsorption on hydrophobic PS-latex especially in the case of electrostatic attraction.

The adsorption on PS-latex was compared with the retention on chromatographic columns and this proved to be an useful tool in the investigation of the importance of the various interactions for protein adsorption such as electrostatic and hydrophobic interactions. The retention of the Savinase variants on the hydrophobic interaction column (Alkyl-Superose®) showed that the retention time was inversely related to the ratio of polar to non-polar surface area on the protein. The retention of the variants on a cation exchange column could not be described satisfactory with the mean surface potential. A first examination of the relation between charge distribution and retention on a negatively charged column showed that strongly localised alterations of the potential could not be expected to lead to differences in retention.

4.1 Introduction

The mechanism of protein adsorption at solid-liquid interfaces depends on both the natures of the interface and that of the protein itself. The effects of varying the experimental conditions, that influence the properties of both the interface and the protein, on the adsorption mechanism has been described in Chapter 3. The experiments reported there allowed us to draw conclusions about the relative importance of the various interactions. In order to relate these interactions to the properties of the adsorbing protein we intended to alter the enzyme characteristics slightly and compare the adsorption with that of the unaltered, so-called "wild type", Savinase under otherwise identical conditions.

This approach was followed before by Norde and coworkers to investigate the adsorption of a number of naturally occurring proteins of similar weight and shape under several experimental conditions [1,2,3]. As those proteins had a different origin and function there was no homology amongst them. Therefore the structural stability, charge and solubility of the proteins differed strongly and this rendered it difficult to relate the changes in adsorption to the characteristics of the protein.

An alternative approach is to study proteins having a similar function in different (but related) organisms. As we are interested in subtilisin-like serine protease adsorption one could consider to study the adsorption of subtilisins found in archaea, bacteria, fungi, yeasts, worms, insects, plants and mammals [4]. However, practical execution of such a study is hampered by the fact that the number of differences in the primary structure of these proteins is still considerable and only a small number of the variants, "engineered" by nature, are available for this kind of adsorption study.

Recent developments in the field of microbiology and biotechnology have solved these practical problems. Protein engineering techniques developed over the years [5,6,7] have enabled the production of relatively large amounts of subtilisin variants with almost any desired alteration in primary structure. These variants proved their relevance in the elucidation of fundamental structure-function relationships in the examination of the mechanism of enzymatic peptide bond hydrolysis [8,9,10,11,12]. In this chapter a small set of closely related Savinase variants will be used to study the relation between the properties and the adsorption of the enzyme at solid-liquid interfaces.

The six variants selected for this adsorption study differ mainly in electrostatic properties from "wild type" Savinase. As far as possible, two parameters were altered independently: the isoelectric point and the number of charged residues. As electrostatic interactions between the interface and the enzyme proved to be important (Chapter 3), a

decrease in isoelectric point would be expected to lead to a decrease in adsorption at a negatively charged interface. Increasing the number of charged residues while keeping the net charge constant would be expected to increase the solubility of the protein and at the same time affect the affinity of the enzyme for the surface. In principle, alterations in the primary structure of a protein can also lead to changes in secondary structure. Such changes could expose the residues which are inaccessible in "wild type" Savinase thereby changing both the solubility and the interaction with the interface. As the occurrence of these changes would obstruct interpretation of the adsorption results, the residues chosen for variation were all located on the surface of the enzyme and they were not part of a structured region of the molecule. In order to verify that the secondary structure of the variants was unaltered we determined the α-helix and β-sheet content of the variants experimentally with circular dichroism spectroscopy. More advanced techniques, such as NMR, that could be used for a more sensitive comparison are not yet fully operational for proteins of this size.

In this study we used liquid chromatography to characterise the selected variants with respect to their adsorption characteristics. In protein chemistry chromatographic techniques are mostly used for purification and identification of components in a mixture. In principle, the technique can also be used for the characterisation of proteins with respect to their adsorption behaviour on different surfaces. The interactions playing a role in the retention mechanism of proteins are numerous and difficult to unravel [13] but they are similar to those of importance for protein adsorption. Therefore, liquid chromatography gives us the opportunity to examine the importance of hydrophobic and electrostatic interactions independently from each other. The use of hydrophobic interaction columns gives the opportunity to study the affinity of a protein for a hydrophobic interface without significant charge-charge interactions.

The main part of this chapter consists of adsorption measurements on polystyrene latices and glass (sections 4.3.3 and 4.3.4). The approach and experimental techniques for the adsorption experiments used in this study are similar to the ones used for the investigation of the Savinase (Chapter 3) and Lipolase (Chapter 5) adsorption mechanism. The adsorption of the Savinase variants was studied for a limited set of different experimental conditions such as pH and ionic strength. The nature of the adsorbent was studied by examining negatively and positively charged polystyrene latices and glass.

4.2 Experimental

4.2.1 Adsorption measurement

Model surfaces
Negatively (indicated by A) and positively charged (indicated by C and D) surfactant-free polystyrene latices were used in this study. The properties of these latices were given before in Table 3.2. Ballotine leadglass beads (ex Tamson, Zoetermeer) were used after cleaning according to the procedure described in section 3.2.1.

Buffer solutions
All adsorption experiments were performed in Borax ($Na_2B_4O_7.10H_2O$) buffer solutions that were adjusted to the desired pH by addition of HCl or NaOH. The ionic strengths of the final buffer solutions were calculated assuming that the borate ion is divalent. The contribution of the ions from the stabilizing buffer that were added together with the enzyme was taken into account. The ionic strength varied only within 10% over the enzyme concentration range used for the measurement of the adsorption isotherms. The calcium concentrations in the final enzyme solutions were up to a maximum of 0.04 mM (molar ratio of enzyme:Ca^{2+} is at least 1:2). The buffers with an ionic strength of 0.1 M were prepared by the addition of NaCl to the 0.05 M ionic strength buffer solutions.

Method
The limited availability of the variants did not allow for the determination of a calibration curve for each individual protein. Therefore the Lowry colouring method was chosen to determine the protein concentration in solution as this method is known to depend mainly on the number of peptide bonds and is virtually independent of the type of protein [14]. As the number of peptide bonds was not varied we assumed that the calibration curves of the variants were identical to the one of Savinase WT. The calibration curve of Savinase WT was described by the equation (see also section 2.3.4):

$$E_{750\,nm} = -3.5092\,[Sav]^2 + 4.9720\,[Sav],\ (0 < [Sav] < 0.34\ mg\ ml^{-1})$$

All adsorption experiments were performed at an ionic strength of 0.05 M, a temperature of 3°C and a contact time of 30 minutes unless stated otherwise.

4.2.2 Savinase variants

The main characteristics of the Savinase variants are summarized in Table 4.1. The residues that were varied were located outside the so-called calcium binding site regions

[19]. Therefore we will assume that the affinity for calcium ions was unaltered. The inserted or deleted *charged* residues were aspartates, glutamates, lysines or arginines as these amino acids are virtually not titrated in the pH range of interest (pH=6 to 10.5).

The variants were stored at concentrations ranging from 11 to 29 mg ml^{-1} in a stabilizing buffer (pH=6.5) consisting of 0.2 M H_3BO_3, 2 mM $CaCl_2$ and 10 mM dimethylglutaric acid. The amount of variant at our disposal was too small to enable enzyme inhibition with PMSF. However, adequate prevention of autolysis could be achieved by lowering the temperature to 3°C and reducing the contact time to 30 minutes (section 3.3.2).

Table 4.1 Savinase variants.

Variant name	Isoelectric point	Charged residues compared to WT	Number of changes
pI10.5EC	10.5	1 less	2
pI9EC	9	1 more	3
pI7HC16	7	16 more	18
pI10LC8	10	8 less	8
pI10HC14	10	14 more	14
pI10HC12	10	12 more	12

* EC stands for equal number of charged residues, HC for higher and LC for lower number of charged residues compared to wild type Savinase.

It was impossible to separate the enzyme from the stabilizing buffer by gel filtration because this normally means a loss of protein of up to 50%. Although the stabilizing solution was diluted at least 100 fold, its presence in the final buffer made it impossible to measure the adsorption at ionic strengths below 0.05 M. Comparison of the adsorption of active Savinase (in the following indicated as "wild type" Savinase or WT), intentionally contaminated by dilute stabilizing solution, with that of purified PMS-Savinase enabled the determination of the influence of components of the stabilizing solution on the adsorption. All variant samples used in this study were monodisperse as checked by Reversed Phase HPLC according to the procedure reported in Appendix 2.5. The variant pI10.5EC was observed to flocculate (reversibly) at moderate enzyme concentration (above 0.13 mg/ml). It was verified that the measured depletion of enzyme from solution at the concentrations used in the adsorption experiments was indeed caused by adsorption and not by centrifugation of small insoluble enzyme agglomerates out of the solution.

Circular dichroism (CD) spectroscopy of variants

Changing several amino acids in a protein does not only induce a (known) change in the primary structure but may also affect the secondary and tertiary structure. In order to investigate possible changes in secondary structure the CD spectra of about 1 mg ml^{-1} enzyme solutions in 0.01 M acetate buffer (pH=5.5) were measured with a Jobin Yvon Dichrograph VI spectroscope. The reference cell was filled with the same buffer and the optical path length of both cells was 0.01 cm. The α-helix and β-sheet contents of the variants were calculated using the program "CONTIN" [15,16]. The protein concentrations necessary for the structure determination were calculated from the ones of the stock solutions that were measured with the Lowry colouring method.

Characterisation of variants using liquid chromatography

The retention times on two FPLC columns were used to characterise the variants. Two columns were chosen: a Mono-S® cation exchange column (ex LKB-Pharmacia) and an Alkyl-Superose® hydrophobic interaction column (ex LKB-Pharmacia). The Mono-S column consisted of a Monobeads matrix [17] based on a beaded hydrophilic resin to which the charged groups ($-CH_2SO_3^-$) are attached. The Alkyl Superose column consisted of neopentyl ($-CH_2-C(CH_3)_3$) groups chemically linked via ether bonds to cross-linked agarose beads (Superose 12). The binary gradients used for elution of the proteins on both columns are shown in Table 4.2.

Table 4.2 Binary gradients used for protein elution on HPLC.

time (min)	Mono-S		time (min)	Alkyl-Superose	
	%A	%B		%C	%D
0	100	0	0	0	100
5	100	0	25	25	75
35	50	50	30	0	100
40	0	100			
45	100	0			

Solution A: 0.01 M NaH_2PO_4, 0.02% sodium azide, pH=6.4
Solution B: 0.3 M NaH_2PO_4, 0.02% sodium azide, pH=6.4
Solution C: 0.01 M $NaH_2PO_4.H_2O$, pH=6.6
Solution D: 0.01 M $NaH_2PO_4.H_2O$, 1.5 M $(NH_4)_2SO_4$, pH=6.6

The HPLC system consisted of two Gilson Model 305 pumps with a 10 SC and a 10 WSC pumphead and a Gilson gradient mixer 811B. The variants were injected via a 100 µl sample loop and they were detected with a variable wavelength UV/VIS detector (ex LKB-Pharmacia, Model 2141) at 280 nm.

4.2.3 Calculation of variant characteristics

Calculated molecular charges as a function of pH

The molecular charges and the number of charged residues of the variants were calculated using Tanford's equation [18] as described before in section 1.2.2 and compared with that of WT Savinase. The calculation of the degree of dissociation of the amino acids is based on the Debye-Hückel theory that determines the Gibbs energy necessary for charging an impenetrable sphere. In this approximation the dissociation constants of the charged amino-acids are corrected for the total charge of the protein molecule assuming this charge to be evenly spread over the spherical molecule. The influence of the microenvironment on the dissociation of the charged residues is not taken into account in this type of calculation. An amino acid is counted as charged (positively or negatively) if the net charge is higher than 0.1 elementary units.

Both the titration curves and the calculated number of charged residues of the variants are shown in Figures 4.1 to 4.4. All calculations were performed assuming that one Ca^{2+} ion occupied the calcium binding site. The temperature was 25°C and the ionic strength was 0.05 M.

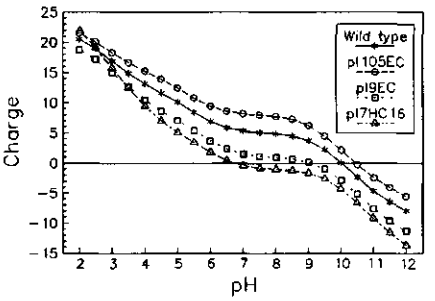

Figure 4.1 Calculated titration curves of Savinase variants with different isoelectric points (symbols do not represent measuring points).

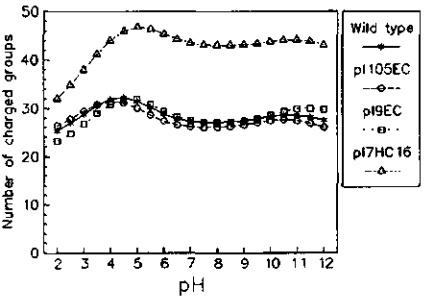

Figure 4.2 Calculated number of charged groups of Savinase variants as a function of pH (symbols do not represent measuring points).

These figures represent two distinct sets of variants selected for this study. One set consists of enzymes which vary in isoelectric points (Figure 4.1), resulting in differences in net charge at a particular pH value. The other set has identical titration curves (Figure

4.3) but a different number of (both negatively and positively) charged residues. One variant, pI7HC16, belongs to both sets as both the isoelectric point is decreased and the number of charged residues is increased.

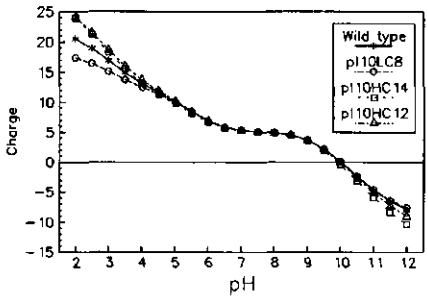

Figure 4.3 Calculated titration curves of Savinase variants with isoelectric points at pH = 10 (symbols do not represent measuring points).

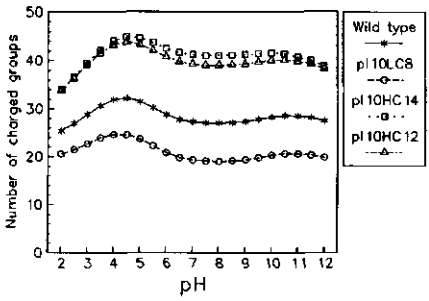

Figure 4.4 Calculated number of charged groups of Savinase variants with isoelectric points at pH = 10 as a function of pH (symbols do not represent measuring points).

Calculated protein surface characteristics

The structure of WT Savinase was determined by X-ray crystallography at 0.14 nm resolution [19]. The structure of the Savinase variants is calculated from the known WT Savinase structure by comparative modelling techniques [20,21,22] as implemented in the Composer module of the Sybyl Molecular Modelling software (ex Tripos Associates Inc., St. Louis, Missouri, U.S.A.). All three dimensional structures are based on energy minimization using the Discovery module [23] of the Biosym Technologies software.

In order to determine the potential at the surface of a protein molecule one has to define the normally irregular surface. The potential is established at regular intervals on the this surface. The first step was to calculate the potentials on a number of regularly spaced grid points. Normally a protein can not be described by a simple geometric structure and therefore the second step consisted of the determination of the surface area. The surface could then be described by a large number of small triangles. The last step is the determination of the potential at the centres of all triangles by interpolation using the previously calculated potentials at the grid points. In the following two paragraphs the calculation procedure of the potential at the grid points and the surface area will be described in more detail.

The potentials were calculated using the Delphi program [24]. This program uses the full three-dimensional structure of the protein and describes the electrical double layer with the non-linear Poisson-Boltzmann equation. The Poisson equation was used as it was the only method available in commercial software even though the region of interest for

the potential calculation is relatively close to the protein surface. The bound charges (charged amino acids), the partial charges (dipole charges within the residues) and the atomic radii were taken from the Discovery atomic force field. One Ca^{2+} ion is assumed to occupy the strongest binding site [19]. The influence of the microenvironment on the dissociation of the charged residues is not taken into account in these calculations. The protein was placed in a grid of 77 by 77 points with a grid size of 0.1 nm. The results of the calculations of the surface potential proved to be independent of the grid spacing. For the dielectric constant of the electrolyte a value of 80 and for the inside of the protein a value of 4 was taken.

The surface of the protein can be defined in various ways as shown in Figure 4.5. The first one is the so-called contact surface and it is defined as the area that is in *direct contact* with a probe sphere with the radius of a water molecule (0.14 nm) as it is rolled over the outer atoms of the protein. The contact surface of the Savinase variants could be described by approximately 14,000 triangles. This representation of the surface is identical to the one used by Haggerty and coworkers [29]. Another way of describing the protein surface is by the so-called water-accessible surface. This smoother surface is 0.14 nm removed from the contact surface. The water-accessible surface of the Savinase variants could be described by approximately 6000 triangles. Both the surfaces and the triangulation were preformed with the molecular surface software package of M.L. Connolly [25] version 1.6. In order to calculate a surface at a particular distance from the contact surface with this program the atomic radii in the protein were enlarged by that distance.

The mean potential at a particular distance from the contact surface was calculated by averaging the potentials at the triangle centres. The potentials shown in the so-called Hammer or Mollweide projection [26] were calculated at all triangle points describing the surface. These projections are a means to visualize the lines connecting points with equal potential at the water-accessible part of the protein similar to the way a world map represents the globe [27]. All atoms on the contact surface of the molecule can be divided into two categories: polar and apolar atoms. This rather rough classification of the atoms of the various amino acids is summarized in Appendix 4.1. The total polar

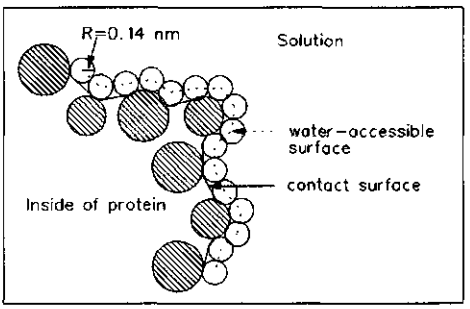

Figure 4.5 Schematic representation of the determination of the surface area of a protein.

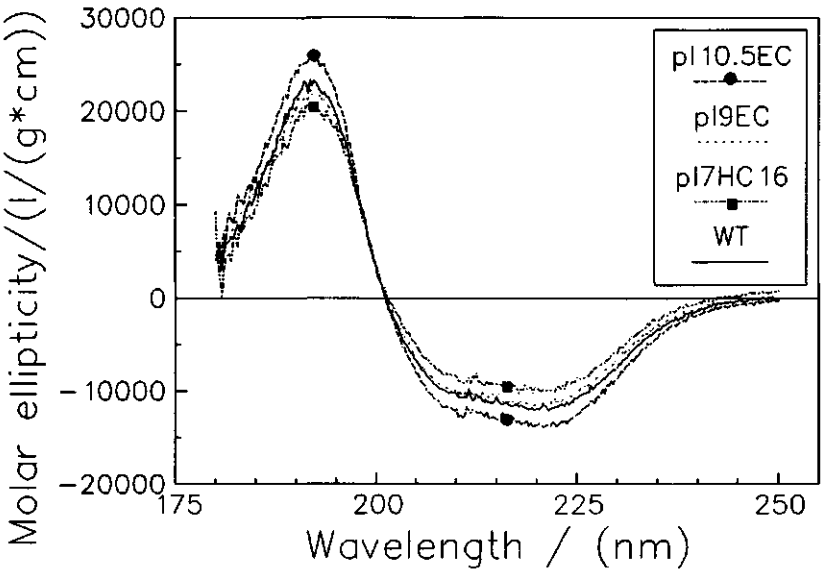

Figure 4.6 CD-spectra of Savinase variants with various isoelectric points.

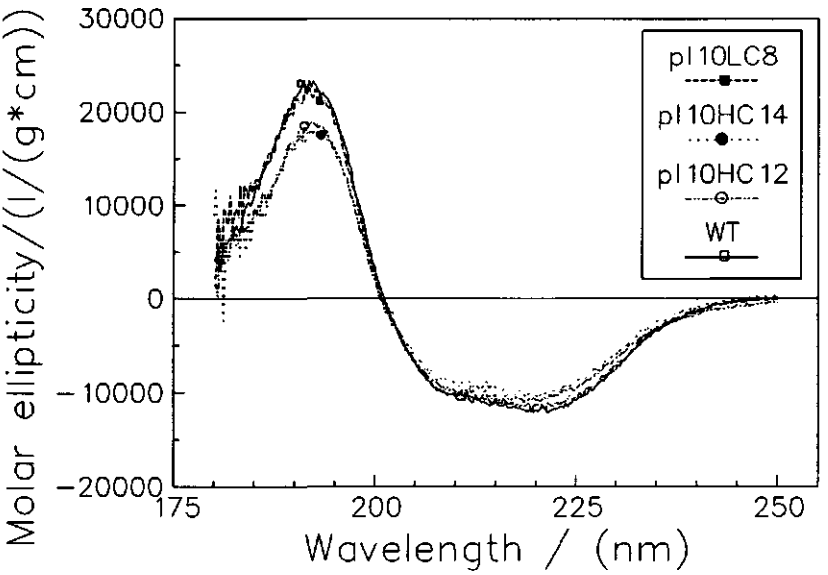

Figure 4.7 CD-spectra of Savinase variants with isoelectric points at pH = 10 and various number of charged residues.

surface area is the sum of the parts of the atoms on the contact surface that are polar. The atoms of the molecular "backbone" are not included in these calculations as they are virtually identical in all variants and we only consider these surfaces for a comparison between the variants.

4.3 Results and Discussion

4.3.1 Characterisation of variants

Secondary structure of Savinase variants

The circular dichroism spectra of the variants are shown in Figures 4.6 and 4.7. The calculated secondary structure is summarized in Table 4.3 together with the variation due to the fitting of the experimental curves. None of the variants showed a significant decrease in secondary structure. Three variants had more structure than WT Savinase namely pI10.5EC had more α-helices and pI7HC16 and pI10HC12 had more β-sheets. The increase in for example α-helix content in pI10.5EC can not be understood as only two residues were altered. Therefore we must realise that an accurate determination of the secondary structure depends not only on the accuracy of the fit but also on the accuracy of the enzyme concentration in the measurement cell and this is very difficult to obtain. Although the structure determination was not very accurate we can conclude that no large scale unfolding was induced by alteration of the residues.

Characterisation of the variants using Mono-S and Alkyl Superose FPLC columns

Two columns were chosen to characterise the Savinase variants with respect to their electrostatic interaction with a negatively charged hydrophilic interface and their interaction with a hydrophobic interface. The negatively charged Mono-S column enabled us to examine the electrostatic interaction between the protein and the surface without interference from the hydrophobic dehydration that partially determined the adsorbed amount on negatively charged PS-latex. The retention on a hydrophobic

Table 4.3 Secondary structure of Savinase variants.

Variant	α-helix %	β-sheet %	random coil %
WT	27 ± 2	49 ± 2	25 ± 3
pI10.5EC	39 ± 4	47 ± 4	15 ± 7
pI9EC	24 ± 2	53 ± 3	23 ± 3
pI7HC16	24 ± 4	65 ± 4	12 ± 7
pI10LC8	25 ± 4	55 ± 4	20 ± 7
pI10HC14	21 ± 3	53 ± 4	26 ± 7
pI10HC12	24 ± 3	60 ± 4	11 ± 6

interaction column (HIC) was thought to be indicative of the hydrophobic interactions which played a role in the adsorption on PS-latex. Electrostatic interactions between the protein and the HIC column can be discounted as the enzymes were eluted at a salt concentration of about 1.4 M while the column is virtually uncharged [28]. In this section we will discuss the relation between the molecular properties and the retention times while in the next one we will discuss the relation between the adsorption characteristics of the variants and the retention times on the columns.

Table 4.4 Retention times on a Mono-S column and the mean surface potentials at the contact and the water-accessible surface of the Savinase variants.

Variant	Retention on Mono-S at pH=6.4 (in mM NaH_2PO_4)	Mean surface potential at contact surface (at pH=7 in mV)	Mean surface potential at water-accessible surface (at pH=7 in mV)
WT	61	23.9	22.8
pI10.5EC	175	22.8	20.9
pI9EC	29	0.8	1.8
pI7HC16	< 10	-16.4	-12.4
pI10LC8	62	24.2	22.3
pI10HC14	91	2.2	18.4
pI10HC12	58	20.1	18.0

Retention on a Mono-S column

The retention on the cation exchange column (Mono-S) correlated qualitatively with the net charge of the variants with altered isoelectric point. However, the variants with identical isoelectric point showed significantly different affinities for the Mono-S column (Table 4.4). This indicates that although the net charge might be important it is not the only factor determining the affinity for the negatively charged surface. This was reported before by Haggerty and coworkers [29] who compared the retention times of a series of proteins with little homology [30]. They showed that for such proteins the retention time correlated very well with the *mean* surface potential of the protein and tentatively explained the phenomenon by the high rotational diffusion coefficient of macromolecules which allows averaging the potential over the whole surface area. The strong correlation was unexpected as the proteins differed significantly in shape and molecular weight and hence in rotational diffusion coefficient. If the correlation could be applied to proteins in general it would certainly have to apply to a set of variants of one protein. We therefore

calculated the mean surface potential of the variants (Table 4.4) in a way identical to the one published by Haggerty [29] namely at the contact surface. As shown in Table 4.4 the relative order of these average values proved to be sensitive to variation of the distance from the contact surface at which the potentials were calculated. This is probably caused by inaccuracies in the calculation of the potential near the charged atoms at the contact surface and possibly also due to the use of the Poisson-Boltzmann equation.

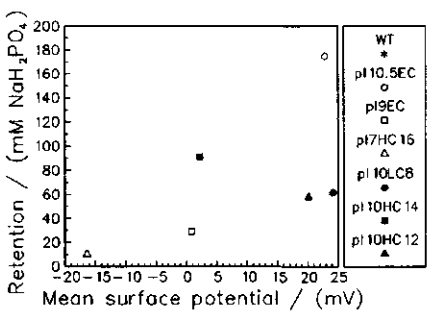

Figure 4.8 The relation between the retention on a Mono-S column at pH=6.4 and the mean surface potential at the *contact* surface at pH=7.

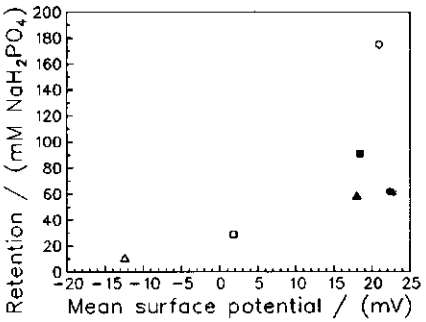

Figure 4.9 The relation between the retention on a Mono-S column at pH=6.4 and the mean surface potential at the *water-accessible* surface at pH=7 (legend in Figure 4.8).

Figure 4.8 shows that the retention times of the Savinase variants studied on a Mono-S column did *not* relate to the mean surface potential at the contact surface in contrast to the results reported by Haggerty et al [29]. However, the mean potential at the water-accessible surface correlated better but still with two exceptions: pI10.5EC and pI10HC12 (Figure 4.9). The fact that Haggerty and coworkers did find a linear relationship with a high correlation coefficient could be caused by a more evenly distribution of charges [46] in the *naturally occurring* proteins, examined by them. Another possible reason could be that the correlation was influenced by the fact that their data set was not completely independent as they used retention times of the same proteins at different pH values. For the understanding of the mechanism by which proteins are retained on a cation-exchange column this result indicates that a more detailed investigation of the distribution of charges will be necessary as neither the net charge nor the average surface potential can explain the experimental retentions (section 4.3.5). Although this shows that we do not yet understand the relation between the molecular properties of the protein and the electrostatic interaction we can still use the experimentally determined retention times as a relative measure for the electrostatic interaction of a protein with an interface.

Retention on an Alkyl-Superose column

The mechanism of retention in hydrophobic interaction chromatography (HIC) is still under discussion in the literature [31,32,33]. The most complete thermodynamic

descriptions of the mechanism were published by Melander et al [34,35,36] and Van Oss and coworkers [37,38]. These take into account the difference in Gibbs energy between the state in which the column is exposed to water and the protein is in solution on the one hand side and the state in which the protein is adsorbed to the column thereby releasing water and salt ions from the column on the other. In this model the influence of the salt concentration is attributed to the corresponding change in surface tension of the water. The columns used for hydrophobic interaction chromatography are less hydrophobic than those for reversed phase chromatography. Therefore, the protein can be eluted by a much more moderate change in the solvent. This type of chromatography does not lead to strong conformational changes in the protein as is concluded from the observation that the activity of several examined enzymes is not influenced by the interaction with the column and the solvents [39,40]. It is therefore often assumed that the retention in HIC is only determined by the water-accessible (outer) part of the protein [41,42].

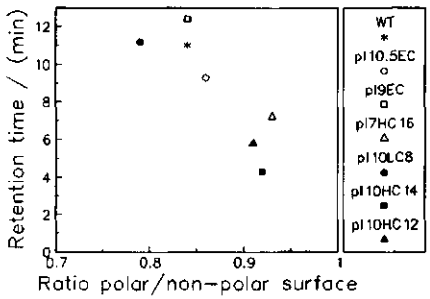

Figure 4.10 The relation between the retention time on Alkyl-Superose column and the ratio of polar to non-polar surface area.

Table 4.5 Retention times on an Alkyl-Superose column and the calculated characteristics of the Savinase variants.

Variant	Retention on Alkyl-Superose at pH=6.6 (min)	Non-polar surface area (nm^2)	Polar surface area (nm^2)	Ratio polar/ non-polar area
WT	11.0	36.90	31.05	0.84
pI10.5EC	9.3	36.88	31.85	0.86
pI9EC	12.4	37.18	31.15	0.84
pI7HC16	7.2	37.29	34.79	0.93
pI10LC8	11.2	37.95	29.99	0.79
pI10HC14	4.3	37.71	35.02	0.92
pI10HC12	5.8	37.50	34.14	0.91

In Table 4.5 the retention times are summarized and the most evident trend is that increasing the number of charged residues leads to a sharp decrease in retention time. In order to quantify this relationship the ratio of polar and non-polar contact surface area of the protein was calculated (Table 4.5). In Figure 4.10 the relation between the retention time and this ratio is shown. It is clear that increasing the polarity of the protein surface decreases the retention on the apolar column. This relation can easily be explained by the fact that increasing the polarity of the protein will be unfavourable for the dehydration that accompanies adsorption on the column. The scatter in the graph can be attributed to the assumptions made to determine of the three dimensional structure of the variant which can cause small inaccuracies in the calculated contact surface area.

4.3.2 The influence of stabilizing buffer on Savinase adsorption

As already stated, the fact that only a small amount of variant was available made it impossible to separate the enzyme from the stabilizing electrolyte solution. In order to determine the influence of the (dilute) buffer we compared the previously reported adsorption of "clean" PMS-Savinase (section 3.3) with that of Savinase in the presence of stabilizing buffer (Figure 4.11). As the influence of the buffer is not negligible we will compare the adsorption of the Savinase variants with that of Savinase WT that was also *not* separated from the same stabilizing solution. The differences between the isotherms in Figure 4.11 are very similar to the earlier reported differences between adsorption measurement in the presence and absence of Ca^{2+} ions (section 3.3.6). Association of calcium with the initially neutral or negatively charged enzyme (at pH=10 and 11 respectively) probably causes the increase in the adsorbed amount at a negatively charged interface.

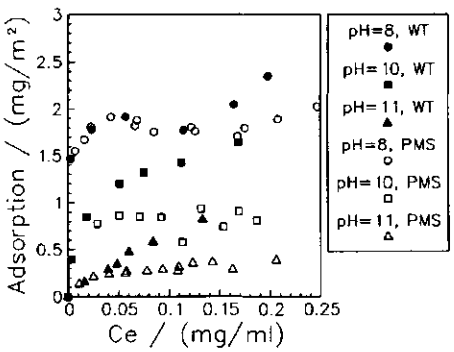

Figure 4.11 Adsorption of Savinase Wild Type from stabilizing buffer compared with PMS-Savinase.

4.3.3 Adsorption of variants with various isoelectric points

Variant pI10.5EC

Changing the isoelectric point of the enzyme leads to a different net charge under all pH conditions (Figure 4.1). The first variant examined, pI10.5EC, has less negatively and more positively charged residues resulting in a higher isoelectric point than the "wild

type" Savinase. We would therefore expect it to adsorb better on negatively charged PS-latex and glass. Figures 4.12 and 4.14 show that this is the case although the increase is much stronger than was expected from the relatively moderate increase in positive charge on the protein. The strong increase is in accordance with the increase in retention on a Mono-S column (Table 4.4).

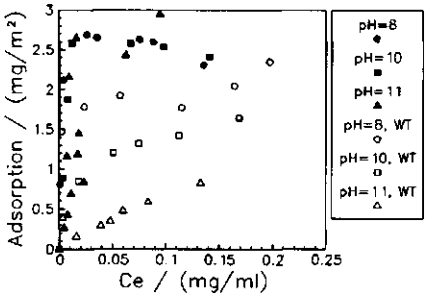

Figure 4.12 Adsorption of Savinase variant pI10.5EC on PS-latex A.

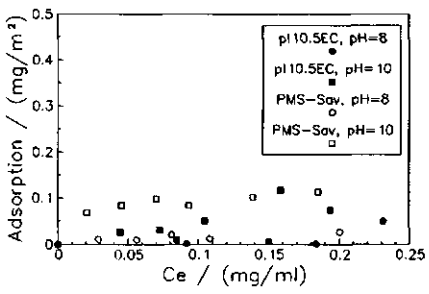

Figure 4.13 Adsorption of Savinase variant pI10.5EC on PS-latex D.

The improvement in adsorption could be complemented by changes in the enzyme other than the net charge such as an increase in hydrophobic interactions induced by the alteration of the primary structure. If this were the case one would expect pI10.5EC to adsorb better on positively charged, hydrophobic, PS-latex. Figure 4.13 shows that at both pH=8 and pH=10 the adsorption of pI10.5EC is slightly lower than that of WT Savinase. This result together with the shorter retention times on the Alkyl-Superose column (Table 4.5) indicates that the hydrophobic interactions have decreased slightly and that it is the increase in electrostatic interaction that has caused the strongly increased adsorption of variant pI10.5EC on the negatively charged latex.

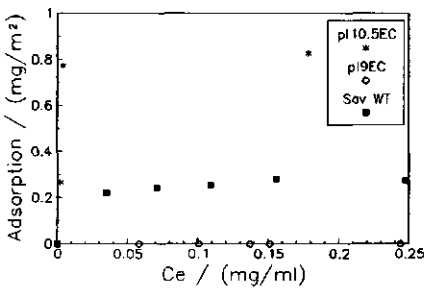

Figure 4.14 Adsorption of Savinase variants with various isoelectric points on glass at pH=8.

Variant pI9EC

Decreasing the isoelectric point of the enzyme towards pH=9 leads to the expected decrease in adsorption on a negatively charged glass, PS-latex A and the Mono-S column as shown in Figure 4.14, Figure 4.15 and Table 4.4, respectively. However, the decrease in adsorbed amount is larger than the difference in net charge on the enzyme at various

pH values as shown in the *calculated* titration curves (Figure 4.1). The most obvious comparison is the adsorption at the isoelectric points of pI9EC at pH=9 and the WT at pH=10. Both proteins are assumed to be neutral but the adsorption of the pI9EC was shown to be much less than that of WT Savinase. This is a first indication that either the calculated net charge is not correct or that the difference in net charge is not the only factor that is changed when going from WT Savinase to pI9EC.

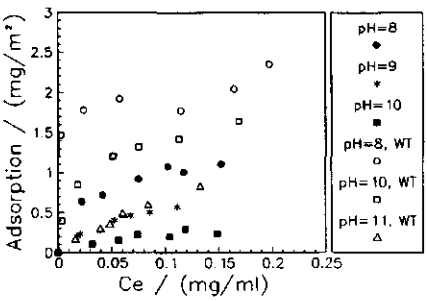

Figure 4.15 Adsorption of Savinase variant pI9EC on PS-latex A.

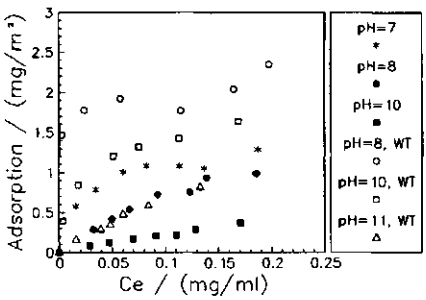

Figure 4.16 Adsorption of Savinase variant pI7HC16 on PS-latex A.

As it was the case for WT Savinase, dehydration of the hydrophobic latex surface is important in the absence of a significant electrostatic attraction. This can be deduced from the negligible adsorption of pI9EC on glass at pH=8 (Figure 4.14), indicating the absence of a significant electrostatic attraction while it does adsorb on negatively charged hydrophobic PS-latex (Figure 4.15).

Variant pI7HC16

This variant has both a lower isoelectric point and a larger number of charged residues. As shown in Figure 4.16, the adsorption on negatively charged PS-latex of pI7HC16 is at any pH much lower than that of WT Savinase just as the retention time on the Mono-S column was shorter. Qualitatively, this is in accordance with the increased negative charge on the variant. Even though Figure 4.16 shows that the adsorbed amounts at the isoelectric points are similar, the adsorbed amount of pI7HC16 at pH=10 is much lower than that of WT at pH=11 whereas both enzymes were thought to have similar net charges at these pH values. As this variant differs also in number of charged residues, the overall lower adsorption could be attributed to the increased hydration of the protein as indicated by the strongly decreased retention on the Alkyl-Superose column (Table 4.5) and discussed in the next section.

Variation in ionic strength

The low isoelectric points of variants pI9EC and pI7HC16 gave the opportunity to verify a previously reported, unexpected observation that the adsorption hardly increased with increasing ionic strength under electrostatically repulsive conditions. In section 3.3.5 it was reported that the adsorption of negatively charged WT Savinase at pH=11 on negatively charged PS-latex A did not increase upon raising the ionic strength from 0.05 M to 0.1 M. As pH=11 conditions were difficult to maintain during the experiment due to the low buffering capacity of the borax buffer system we used low isoelectric point variants to examine this phenomenon further at lower pH values.

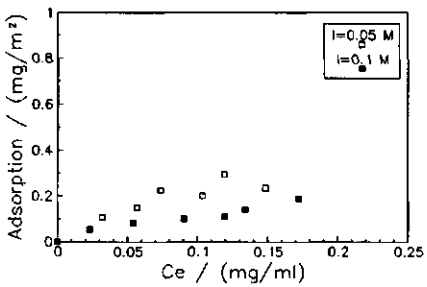

Figure 4.17 Adsorption of Savinase variant pI9EC at pH=10 and various ionic strengths on PS-latex A.

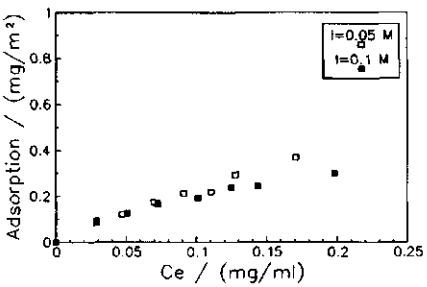

Figure 4.18 Adsorption of Savinase variant pI7HC16 at pH=10 and various ionic strengths on PS-latex A.

In figures 4.17 and 4.18 it is shown that increasing the ionic strength from 0.05 M to 0.1 M did again not lead to increased adsorption under electrostatically repulsive conditions. The adsorption of pI9EC actually decreased. These experiments indicate that the previously reported observations for WT Savinase were not caused by artifacts but are one of the characteristics of Savinase adsorption. However, these experiments did not lead us to an explanation for this phenomenon.

4.3.4 Adsorption of variants with varying number of charged residues

Changing the number of charged residues while keeping the net charge constant can influence the adsorption characteristics of the enzyme in two different ways. This becomes obvious when we realize that adsorption can be described as the replacement of solvent molecules at the interface by the protein with simultaneous replacement of solvent molecules from the contact area of the protein by the interface. If we alter the protein molecule we will not only alter the interaction between the protein and the interface but also the solvation of the protein. A distinction between both consequences can not be made unambiguously but in the case that only the solvation of the protein is altered one would expect the adsorption to change irrespective of the surface characteristics.

If solvation of the protein contributes considerably to the adsorption when compared to protein-interface interactions, one would expect the number of charged residues to be inversely proportional to the affinity of the isotherm and possibly also to the plateau value. The effect on adsorption would also be independent of the properties of the interface. We investigated the importance of solvation for Savinase adsorption using three variants which had a relatively large number of altered residues. This was necessary to maximise possible effects on adsorption as the number of charged groups in WT Savinase is already quite large (see also Figure 4.4).

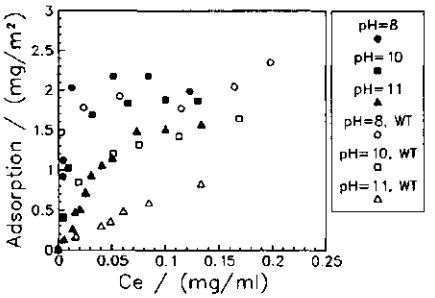

Figure 4.19 Adsorption of Savinase variant pI10LC8 on PS-latex A.

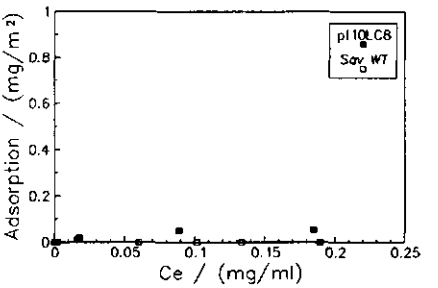

Figure 4.20 Adsorption of Savinase variant pI10LC8 on glass at pH=10.

In Figure 4.19 the adsorption of pI10LC8 is compared with that of WT Savinase on negatively charged PS-latex A. The adsorption increased at all pH values but most significantly at pH=10 and 11. This enhancement was not independent of the surface properties as neither the adsorption on glass (Figure 4.20) nor the retention on the Mono-S or the Alkyl-Superose column increased significantly (Table 4.5). This surface-dependence made it unlikely that differences in hydration determined the higher adsorption of pI10LC8 on PS-latex A.

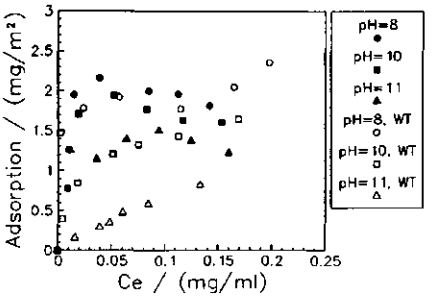

Figure 4.21 Adsorption of Savinase variant pI10HC14 on PS-latex A.

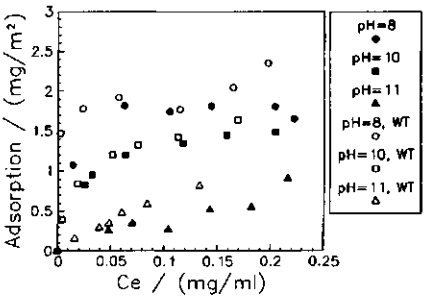

Figure 4.22 Adsorption of Savinase variant pI10HC12 on PS-latex A.

Surprisingly, pI10HC14 (Figure 4.21) also showed an enhanced adsorption although the retention on the hydrophobic interaction column was much weaker than that of WT Savinase. This indicated that altering the enzyme as we did, influenced the hydration conform expectations but that this change did not result in a decreased adsorption on hydrophobic, negatively charged PS-latex. This became even more obvious when looking at the adsorption of pI10HC12 in Figure 4.22. Although the physico-chemical characteristics of pI10HC12 and pI10HC14 (and the retentions on HIC) are very similar, the adsorption differed strongly, especially at pH=10 and 11.

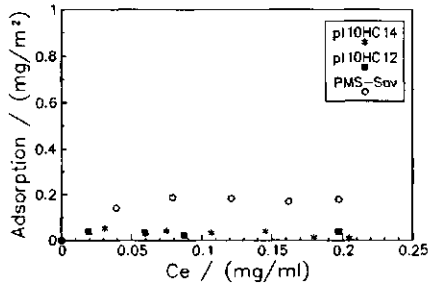

Figure 4.23 Adsorption of Savinase variants with increased number of charged residues on PS-latex C at pH=8.

On positively charged PS-latex C, under repulsive electrostatic conditions, both pI10HC14 and pI10HC12 adsorbed less than WT Savinase (Figure 4.23). The decreased adsorption of pI10HC14 again indicated that the higher adsorption on negatively charged PS-latex A was not caused by increased hydrophobic interaction and that the differences have to be explained by differences in electrostatic characteristics of the variants.

We can now conclude from both sets of variants that the description of the protein and/or the adsorption mechanism is not yet complete. Experiments with the set of mutants with different isoelectric points showed that the net charge is an important variable. Qualitatively the influence of electrostatic interaction on adsorption is confirmed. However, using the net charges calculated with Tanford's equation we can not explain the differences in the adsorbed amount and the pH dependencies. This conclusion is confirmed by the fact that the variants which were expected to have identical net charges had different electrostatic interactions with the interface. Again we must conclude that either the net charges differ from the ones calculated with Tanford's equation or that it is not only the net charge but for example also the distribution of charges that is of importance for adsorption. In the next section the latter possibility will be examined by a qualitative comparison of the potential distribution of the variants with identical isoelectric point. We also conclude that solvation of the protein is important for adsorption but its influence is only visible in absence of electrostatic interactions.

4.3.5 Molecular cartography of the electrostatic potential on the protein surface

The results reported in Chapter 3 and this chapter suggested that electrostatic characteristics other than the net charge or the mean surface potential of the enzyme could determine the interaction with a charged interface. In this section the possible contribution of charge, and hence potential, *distribution* to the interaction is examined. Charge distribution, or "mosaic" charges, are thought to be important if the differences in interaction energy between various orientations of the protein are large relative to the energy of the Brownian motion. Another condition is that the molecule must rotate quickly enough, compared to the time scale of the approach of the interface, to adsorb in the orientation with the lowest interaction energy. If the latter condition is not fulfilled it is not useful to consider potential patches as the enzyme could adsorb in all orientations with a negative, but not necessarily the lowest, interaction energy. A simple calculation of the angular rotation of the enzyme whilst interacting with the interface can be used to verify that this condition is fulfilled.

It was assumed that electrostatic interaction between the enzyme and the interface occurred only within a distance of the order of twice the Debye length ($2\kappa^{-1}$). The translational and rotational diffusion coefficients of a spherical molecule with a radius of 2.3 nm were estimated to be $9.5\,10^{-11}$ $m^2\,s^{-1}$ and $1.3\,10^{7}$ s^{-1}, respectively according to the Stokes-Einstein equation. The time necessary for Savinase to diffuse over the distance of $2\kappa^{-1}$ (about 2.6 nm and 6 nm at ionic strengths of 0.05 M and 0.01 M, respectively) is $3.6\,10^{-8}$ and $2\,10^{-7}$ seconds while the r.m.s. angle over which the enzyme is rotated is 80° and 190°, respectively [43]. Although not the complete molecule can interact electrostatically with the surface during the approach of the interface the rotational motion is fast enough for the charge distribution on the protein to be important.

In this section a first impression of the importance of the electrostatic potential distributions will be obtained by visualisation of the potential maps of the four variants with identical net charge, pI10LC8, pI10HC14, pI10HC12 and Savinase WT. In principle the calculated potentials, reported here could be used to determine the orientation-dependent interaction energy of the variants with a negatively charged surface, in a similar way as recently reported by Yoon and Lenhoff [44,45] for RNase. However, in this section the discussion will be limited to a *qualitative* examination of the relation between the potential distributions and the retention times on the cation exchange column. We will merely answer the following question: do the differences in the charge distribution on the four variants (with identical net charge) explain the strong retention of pI10HC14 on the one hand compared to the retentions of WT Savinase, pI10LC8 and pI10HC12 on the negatively charged column on the other hand? The comparison was

restricted to the experimental data on the interaction with the Mono-S column as these were the most straightforward results available representing the electrostatic interactions between the enzyme and the surface at low surface coverage.

The ionic strength in the potential calculations was chosen to be 0.01 M to enlarge the differences between the variants. This choice is rather arbitrary as the enzyme was eluted from the Mono-S column with a gradient in the salt concentration. The distances between the individual charges on the adsorbent, about 1.3 nm, were equal to or smaller than the Debye length. Therefore, the charge at the interface was assumed to be homogenously distributed at a distance of 0.29 nm, that is the radius of the charged group on the latex surface, the sulphate ion, (Figure 4.24). As this was the shortest possible distance between the enzyme and the surface, the potentials were calculated at 0.29 nm from the contact surface of the enzyme.

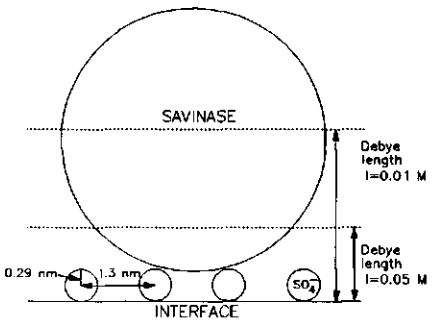

Figure 4.24 Schematic comparison of the dimensions of the enzyme, the charged sulphate groups and the Debye length.

The Hammer projections of the potentials at 0.29 nm from the contact surface of the Savinase variants are shown in Figures 4.27 to 4.30. The total surface area of the variants with a specified potential is shown in Figure 4.26 and we can conclude from this graph that the rather arbitrary choice of equal-potential-lines does not influence the comparison between the variants. For the interpretation of the potential maps the sizes of the potential patches must be related to the sizes of the surface areas that were in contact with the interface. In Chapter 3 the dehydrated surface area on the PS-latex was calculated to be of the order of 4.4 nm^2, corresponding to the area of a circle with a radius of 1.2 nm. Assuming that the size of this patch was similar for the various surfaces, the area on the protein in "contact" with the interface could be determined as illustrated in Figure 4.25. This simple calculation showed that the potential had to be averaged over a relatively large area, corresponding to 17% of the total surface of the enzyme. As the software was not yet available to perform this averaging over a part of the molecular surface we concluded that

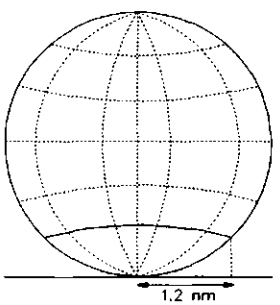

Figure 4.25 Surface area on enzyme in interaction with interface (dimensions are not scaled).

only large positive and negative potential patches should be considered as important for the interaction with an interface as the small patches will "disappear" upon averaging. From the large size of the patches that are thought to be important for adsorption it could be concluded that only quite drastic alterations of the charge distribution were expected to lead to altered adsorption characteristics.

Comparison of the potential maps of the four variants (Figures 4.27 to 4.30) shows that the negative potentials are restricted to a relatively concentrated area except in the case of pI10HC12. The long retention of pI10HC14 on a negatively charged column could be attributed to the large area of positive potential in the upper left quadrant. However, at the moment it can not be proven that the potential *distribution* causes the differences in retention on an ion-exchange column as for example the seemingly small differences in distribution between the potential maps of pI10LC8 (Figure 4.28) and pI10HC14 (Figure 4.29) contrast their significant difference in retention time. In order to obtain more certainty about the importance of potential distribution it will be necessary to calculate the orientation dependent interaction energy of the enzyme with a charged interface.

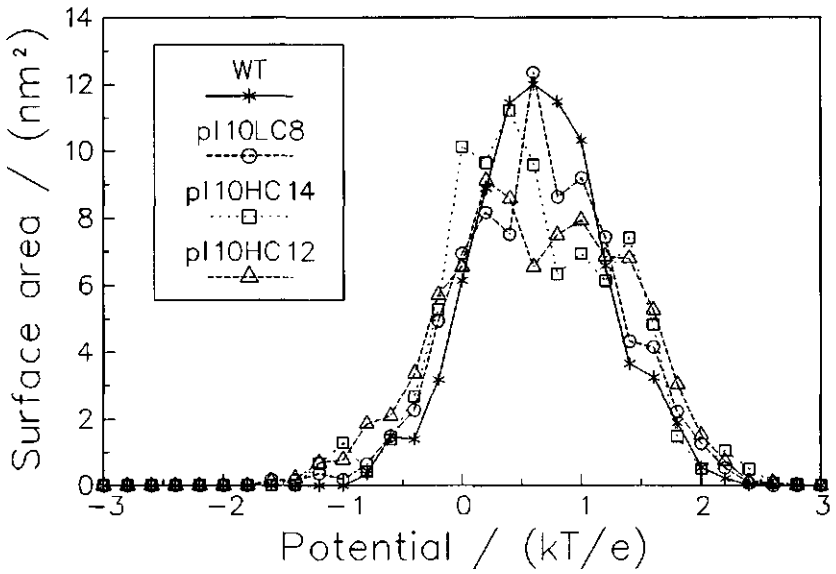

Figure 4.26 Distribution of surface potentials on the Savinase variants.

Looking again at Figure 4.26 it must be noted that it showed an unexpected phenomenon when relating the induced alterations in the protein to the potential maps. Even though we altered a large number of residues, it did not result in a significant difference in surface area with higher or lower potential. This indicated that the potential originating

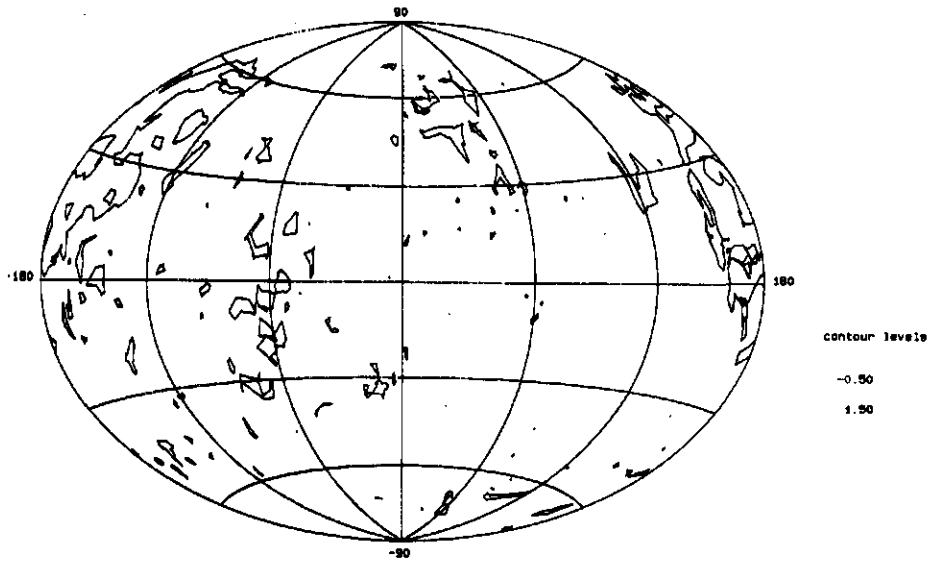

Figure 4.27 Hammer projection of potential map of WT Savinase at 0.29 nm from the contact surface. Contour potential levels at -0.5 kT/e and +1.5 kT/e.

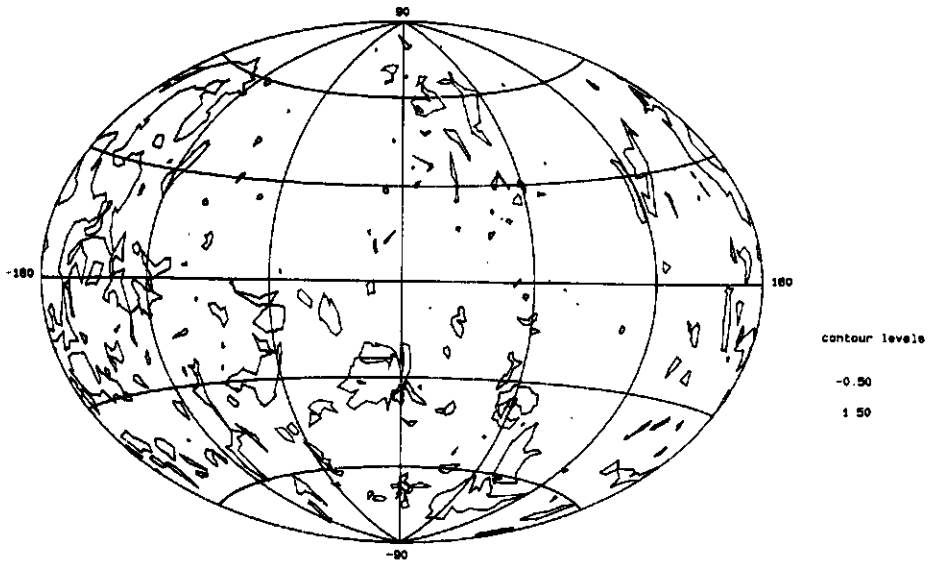

Figure 4.28 Hammer projection of potential map of pI10LC8 at 0.29 nm from the contact surface. Contour potential levels at -0.5 kT/e and +1.5 kT/e.

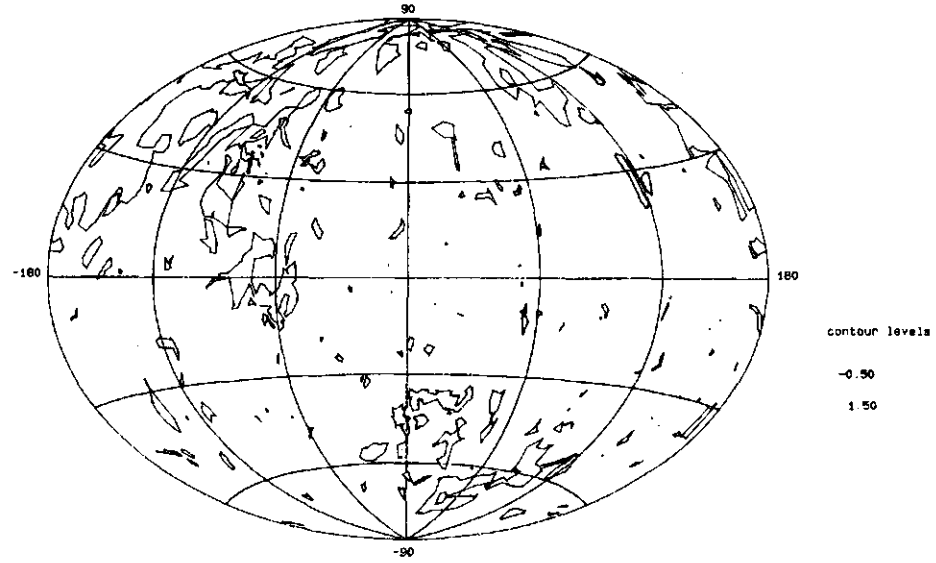

Figure 4.29 Hammer projection of potential map of pI10HC14 at 0.29 nm from the contact surface. Contour potential levels at -0.5 kT/e and +1.5 kT/e.

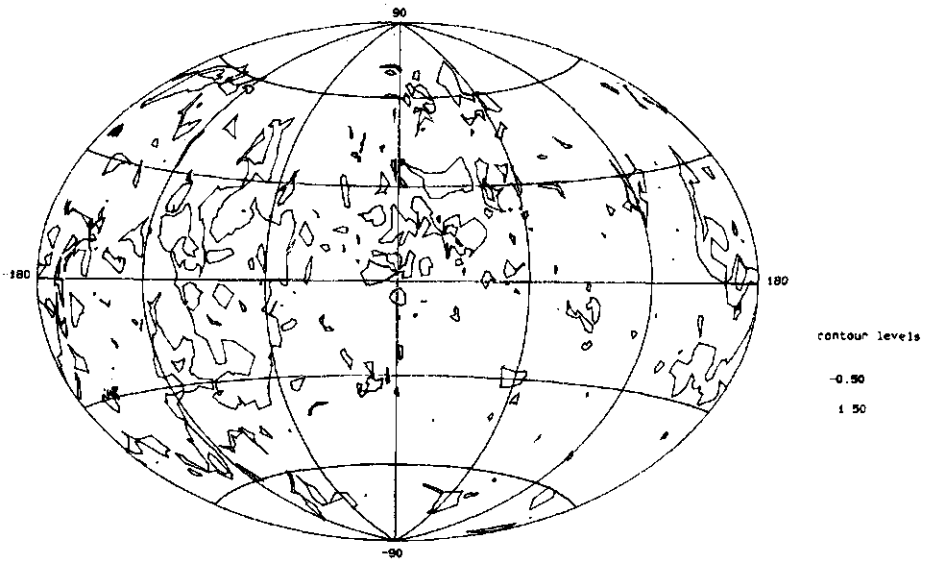

Figure 4.30 Hammer projection of potential map of pI10HC12 at 0.29 nm from the contact surface. Contour potential levels at -0.5 kT/e and +1.5 kT/e.

from inserted positively and negatively charged residues compensated each other in an effective way. The compensation is probably caused by the fact that the charged groups are located close together relative to the range of the interaction. Therefore, we conclude not only that the differences between the variants were much less significant than we expected but also that electrostatic interaction between the dissociating amino acids on the protein surface could not be neglected. This implied that the net charge could also depend on the positions of the charged residues even though as many positively and negatively charged residues were inserted. However, neither the titration curves calculated with Tanford's equation nor the Delphi potential calculation took into account the microenvironment of the charged residues. Tanford's equation took into account the influence of net charge of the protein on the dissociation of a particular residue but not the position of the charges. The Delphi program did not include the influence of other charges on the dissociation of one residue.

This leads to the conclusion that in order to relate the electrostatic properties such as total charge and charge distribution of man-made variants to the structure of the protein it is necessary to account for the microenvironment of the charged residues and its influence on the dissociation of those residues. The explanation for the fact that this necessity did not occur before when titrating *natural* proteins is probably that the clustering of like charges is normally avoided in these proteins [46]. When positively and negatively charged residues are located close together the dissociation will be enhanced and, as most charged residues are either strongly acidic or strongly basic, this will not lead to detectable alteration of the dissociation constant. However, in the case of like charges in close proximity they will reduce each other's dissociation, thereby preventing the build-up of a high local electrostatic potential which could destabilise the protein.

4.4 Conclusions

The differences in electrostatic interactions dominate the adsorption of the various Savinase variants on negatively charged PS-latex. Hydration of the enzyme is less important for the adsorption on hydrophobic PS-latex especially in case of electrostatic attraction. The electrostatic characteristics of the variants can not be described completely by the net charge of the protein as calculated with Tanford's equation or the mean surface potential. Altering the primary structure of the protein brings about changes in electrostatic properties such as the net charge which can not be described properly without taking into account the position of the charged residues.

The retention on chromatographic columns is an useful tool in the investigation of the various important interactions for protein adsorption such as electrostatic and hydrophobic interactions. The retention of the Savinase variants on the hydrophobic interaction column (Alkyl-Superose) showed that the retention time was inversely related to the ratio of polar to non-polar surface area on the protein. The retention of the variants on a cation exchange column could not be described satisfactory with the mean surface potential. A first examination of the relation between charge distribution and retention on the column showed that strongly localised differences could not be expected to lead to differences in adsorption on a charged interface.

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APPENDIX 4.1

POLARITY OF ATOMS IN AMINO-ACIDS

The distinction made between polar and non-polar atoms is shown in Table 4.6. This rough division is necessary for the calculation of the ratio of polar and non-polar surface area on a protein (section 4.3.1).

Table 4.6 Categorization of the atoms in amino-acids as polar and non-polar atoms.

amino-acid	polar atom	non-polar atom	amino-acid	polar atom	non-polar atom
Ala		Cβ	Lys	Cε, Nζ	Cβ, Cγ, Cδ
Arg	Nε, Cζ, Nη1, Nη2	Cβ, Cγ, Cδ	Met		Cβ, Cγ, Sδ, Cε
Asn	Cγ, Oδ1, Nδ2	Cβ	Phe		Cβ, Cγ, Cδ1, Cδ2, Cε1, Cε2, Cζ
Asp	Cγ, Oδ1, Oδ2	Cβ	Pro		Cβ, Cγ, Cδ
Cys		Cβ, Sγ	Ser	Oγ	Cβ
Gln	Cδ, Oε1, Nε2	Cβ, Cγ	Thr	Oγ1	Cβ, Cγ2
Glu	Cδ, Oε1, Oε2	Cβ, Cγ	Trp	Nε1	Cβ, Cγ, Cδ1, Cδ2, Cε1, Cε2, Cζ1, Cζ2, Cη
His	Nδ1, Cδ2, Cε1, Nε2	Cβ, Cγ	Tyr	Oη	Cβ, Cγ, Cδ1, Cδ2, Cε1, Cε2, Cζ
Ile		Cβ, Cγ1, Cγ2, Cδ	Val		Cβ, Cγ1, Cγ2
Leu		Cβ, Cγ, Cδ1, Cδ2			

Note: the position of the atoms relative to the backbone Cα is indicated by a Greek letter.

CHAPTER 5

LIPOLASE™ ADSORPTION ON POLYSTYRENE LATICES AND GLASS

Abstract

Lipolase is the trade name for a fungal lipase that is currently used in fabric washing detergent formulations. Lipases are enzymes that can catalyze the hydrolysis of ester bonds in for example triacylglycerol molecules. An important characteristic of these enzymes is that they are water-soluble whereas their substrate is water-insoluble. The adsorption of Lipolase was studied on several polystyrene latices and glass in order to determine which interactions are of importance for the adsorption on solid-liquid interfaces.

Electrostatic interaction and dehydration of hydrophobic surfaces are the main driving forces for Lipolase adsorption. Under attractive electrostatic interaction between the surface and the enzyme the plateau value of the isotherm corresponds to a monolayer coverage (2.3 mg m^{-2}). Under experimental conditions in which dehydration of the hydrophobic surface is almost compensated by electrostatic repulsion the lateral repulsion between the adsorbed enzymes becomes important and determines the surface coverage of Lipolase. The adsorption mechanism of Lipolase is comparable with that of Savinase. The difference between Lipolase adsorption and that of proteins examined in the literature is that Lipolase adsorbs much less on hydrophobic interfaces. It is concluded that the dehydrated surface area was relatively small and consequently the enzyme did not unfold significantly upon adsorption.

5.1 Introduction

Lipolase is the commercial trade name [1] for the extracellular lipase from the thermophilic fungus *Humicola Lanuginosa* S-38 [2], expressed in *Aspergillus oryzae*. Lipolase is currently available for application in fabric washing detergent formulations to improve fatty-soil removal [3]. Lipases (or glycerol ester hydrolases; enzyme classification number 3.1.1.3) can catalyze both the hydrolysis and the formation of the ester bonds in e.g. triacylglycerol molecules. In the natural environment lipases are very important enzymes that are found in animals, plants and micro-organisms such as yeasts, fungi and bacteria [4,5].

One of the most important characteristics of the catalysis of hydrolysis reactions by lipases is that the enzyme is water-soluble while the substrate, the triacylglycerol, is water-insoluble. The lipolytic activity is reported [6,7,8,9] to increase significantly in the presence of an interface [10,11,12]. This so-called interfacial activation is suggested to be caused by several factors such as a high substrate concentration at the interface [9], a better orientation of the scissile ester bond [13], a better orientation of the enzyme and a conformational change in the enzyme [12,14]. The latter possibility was examined for the lipase the fungus *Rhizomucor miehei*, whose X-ray structure was determined with and without a substrate analogue. The active site Serine residue that is part of the catalytic triad, serine..histidine..aspartic acid (similar to the catalytic site of serine proteases) [15], was shown to be buried under a short helical fragment of a long surface loop. In the lipase-inhibitor complex the "lid" is opened and moved away from the active site. Therewith the hydrophobic residues of the cleft around the active site residues are exposed [16]. Although a X-ray structure of Lipolase is not yet available, it is thought to resemble the lipase from *Rhizomucor miehei* as the primary structures show a significant homology.

The function and working mechanism of lipases indicate that adsorption on the water-substrate interface is a crucial step in the mechanism of enzymatic fat hydrolysis. Investigation of enzyme adsorption on a hydrolysable interface is complicated by the formation of reaction products. In the case of lipase adsorption on a triacylglycerol-water interface, diacylglycerol, monoacylglycerol, glycerol and fatty acids are formed. As most of these reaction products are surface active, they can alter the physico-chemical properties of the interface, compete with the enzyme for the surface and at the same time interact with the lipase in solution. The progress made in the investigation of these aspects of the working mechanism of lipases was recently reviewed by Piéroni et al [17].

In line with our investigation of the hydrolysis of peptide bonds of immobilized proteins by proteases (Chapter 3), we examined the adsorption on a non-hydrolysable interface. In this chapter the adsorption of Lipolase on Polystyrene latices and glass from buffered solutions will be reported. In contrast to proteases, lipases do not auto-digest; therefore, inhibition of Lipolase was not necessary. We used the Lowry colouring method to determine the lipase concentrations. However, this method could not be used to determine concentrations as low as 1 $\mu g\,ml^{-1}$ which are relevant for the practical application of these enzymes. Therefore, we examined the possibility of using the Radio-active Tracer Technique (RTT) and radio-actively labelled [^{3}H]-Lipolase for this concentration range. A detailed description of both experimental techniques was given in Chapter 2 of this thesis.

5.2 Experimental

5.2.1 Materials

Lipolase

Lipolase was purified from commercial preparation (ex. NOVO-Nordisk) by elution on a Phenyl-Sepharose column in a pH=8 TRIS-buffer. The eluent was dialysed overnight against water and freeze-dried afterwards. The freeze-dried enzyme was stored at 5°C and the solutions were freshly prepared each day. The main physical properties of Lipolase are summarized in Table 5.1.

Radio-actively labelled [^{3}H]-Lipolase

Radio-actively labelled [^{3}H]-Lipolase was prepared by a reductive methylation reaction identical to the one used for the preparation of [^{3}H]-Savinase [18]. Two batches of radio-active material were examined. The batch indicated by "11-'88" was made by the "old" radio-active labelling method. Batch "3-'91" was made according to the "new" labelling method that was developed for the labelling of Savinase and PMS-Savinase (section 2.3.2). The properties of the radio-active labelled enzyme were determined with Reversed Phase HPLC using a Milton Roy pump system and UV 214 nm detector or Ramona radio-activity detector (Appendix 2.5). In one case the determination of radio-activity was performed discontinuously by liquid scintillation counting (using Emulsifier Scintillator 299 TM) after collection of one-minute-fractions.

Table 5.1 Physical properties of Lipolase.

Molecular weight	27000 g mol^{-1}
Iso-electric point	pH=4.3
Dimensions	5.3 x 5.0 x 4.1 nm^3

Surfaces and buffer solutions

The surfactant-free latices, the glass beads and the buffer solutions used in this study were identical to the ones used for Savinase adsorption measurements and were described in section 3.2.1 and 3.2.2. Comparison of the Lipolase adsorption from solutions with low and high Borax concentrations but identical ionic strength and pH showed that Borax buffer ions did not influence the adsorbed amount.

5.2.2 Adsorption measurement

The adsorption was determined by depletion measurements after 1 hour contact time. Both the principles and the experimental procedures for the adsorption and protein concentration determination were described in Chapter 2.

The Lipolase concentrations were determined with the Lowry colouring method and in one experiment with the RTT method. In order to determine the concentration with the Lowry colouring method in calcium-containing solutions an additional filtration step was necessary to remove the $CaCO_3$ and $Ca(OH)_2$ after the second colouring step. The filtration step did not influence the concentration determination. The Lowry colouring calibration curve for Lipolase is shown in Figure 5.1. The calibration curve could be fitted to the equation:

$$E_{750\,nm} = -1.864\,[Lip]^2 + 3.101\,[Lip], \text{ with } 0 < [Lip] < 0.5 \text{ mg ml}^{-1}.$$

The concentrations of the Lipolase solutions used for the Lowry colouring calibration curve were determined from the weight of freeze-dried material. In order to check if the freeze-dried material did not contain too much water, the same solutions were used for a calibration curve of UV extinction at 280 nm. The UV 280 nm extinction coefficient that resulted from a linear fit of this curve is equal to $1.128\ l\,g^{-1}\,cm^{-1}$ (Figure 5.2). Comparison of this value with the theoretical value of $1.133\ l\,g^{-1}\,cm^{-1}$ [19] indicated that the freeze-dried material did not contain an appreciable amount of water.

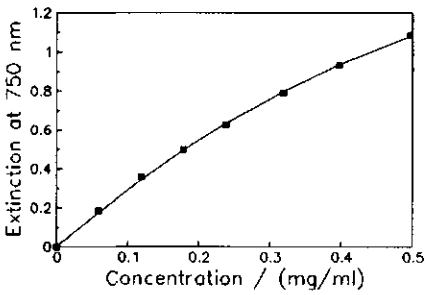

Figure 5.1 Lowry colouring calibration curve of Lipolase with 1 cm optical path length.

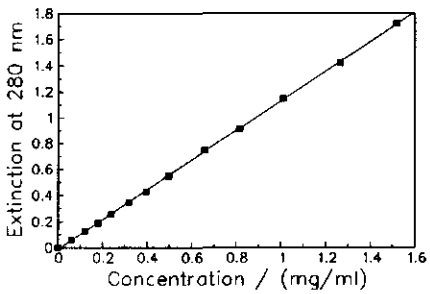

Figure 5.2 Calibration curve of UV extinction at 280 nm of Lipolase with 1 cm optical path length.

The execution of the radio-active tracer technique is similar to the procedure reported in Chapter 2 for Savinase concentration determination. The radio-activity of the 0.1 to 0.2 ml samples of the protein solutions was measured using Emulsifier Scintillator 299 TM as the scintillation liquid and the TRI-CARB Analyzer (type 1900 CA or 2000). Before mixing 7 ml of the scintillation liquid with the enzyme solution, the latter was diluted with water up to 1 ml to achieve optimal solubilization of the enzyme in the emulsion during counting. All samples were counted twice for 10 minutes. The minimum counting rate had to be as high as 150 cpm (counts per minute) in order to get reliable results.

5.2.3 Experimental determination of titration curve

The experimental titration curve of a 3 mg ml^{-1} Lipolase solution was measured with a Radiometer VIT90 video titrator and a Radiometer combined glass electrode GK 2401C. The enzyme was added to a 0.05 M KCl solution and titrated from low to high pH with 0.05 M KOH and vice versa with 0.1 M HCl. The temperature was kept constant at 25°C. CO_2 was removed before titration by leading argon through all solutions for at least 30 minutes. The added amount of titrant was corrected for the experimentally determined uptake of 0.05 M KOH by a 0.05 M KCl solution under identical conditions. The isoelectric point of Lipolase at pH=4.3 was taken as the point of zero charge.

5.3 Results

5.3.1 Titration curves of Lipolase

The experimental titration curves for Lipolase are shown in Figure 5.3. The small difference between the forward and backward titration was probably caused by the uptake of CO_2 from the air at high pH values. Comparison of the reference titration curve of 0.05 M KCl solution with the theoretically expected one (Figure 5.4) indicated that the electrode did not give correct results at pH values above 10.

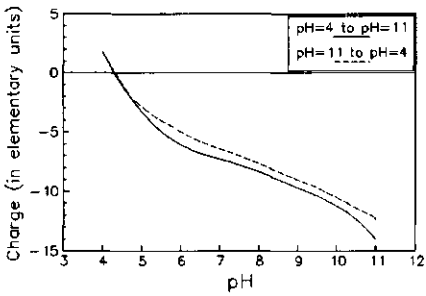

Figure 5.3 Experimental titration curves of Lipolase. Conditions: I=0.05 M, T=25°C, I.E.P.=4.3.

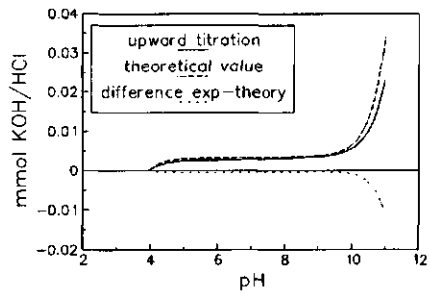

Figure 5.4 Reference titration curves of KCl-solution. Conditions: I=0.05 M, T=25°C.

5.3.2 Variation of surface characteristics

In Figure 5.5 the adsorption isotherms on three differently charged PS-latices at pH=8 are shown. The adsorption isotherm on a positively charged surface was high affinity and the plateau value of the isotherm was 2.3 mg m^{-2}. This value is of the order of monolayer coverage (close-packed monolayer≈3 mg m^{-2}) assuming that Lipolase is a spherical

molecule with a radius of 2 nm. When the enzyme and the surface were both negatively charged the adsorption was much less than a monolayer and decreased with increasing negative charge on the surface. This shows clearly that electrostatic interaction between the surface and the enzyme is an important driving force for the adsorption of Lipolase on Polystyrene latices. However, other interactions must play a role also, as the adsorption on a moderate negatively charged latex (B) is not negligible.

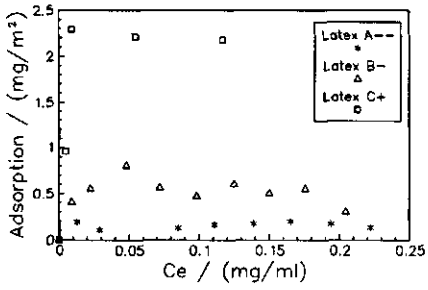

Figure 5.5 Lipolase adsorption isotherms on differently charged PS-latices. Conditions: pH=8, I=0.01 M, T=3°C.

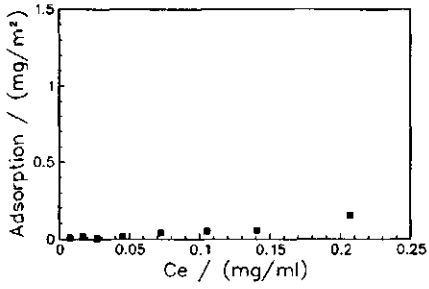

Figure 5.6 Lipolase adsorption isotherm on glass. Conditions: pH=8, I=0.05 M, T=30°C.

The fact that the adsorption on negatively charged hydrophobic PS-latex is significant indicates that either dehydration of the hydrophobic interface and/or unfolding of the enzyme is an important driving force. We examined the adsorption on glass, a hydrophilic negatively charged interface and the results are shown in Figure 5.6. The adsorption on a hydrophilic interface is negligible and this indicates that dehydration of the hydrophobic surface is the important interaction causing the significant adsorption on latex B (Figure 5.5). Another conclusion is that unfolding of the enzyme together with dehydration of the hydrophobic patches on the enzyme is not enough to overcome the electrostatic repulsion between the enzyme and the glass surface.

5.3.3 Variation of pH

Increasing the pH from 8 to 10 led to a lower plateau value on PS-latex (B) as shown in Figure 5.7. The negative charge of the Lipolase molecule is 3 elementary units (30%) higher at pH=10 than at pH=8 (Figure 5.3). This increase in negative charge increased the repulsion between the

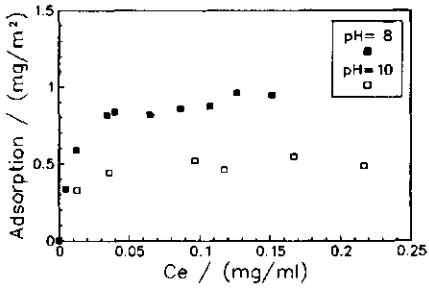

Figure 5.7 Lipolase adsorption isotherms at different pH values on PS-latex B. Conditions: I=0.01 M, T=30°C.

enzyme and the surface and therefore lowered the plateau value of the isotherm. Increasing the net charge on the enzyme did not only increase the repulsion between the enzyme and the surface but also the lateral electrostatic repulsion between the enzymes in the surface layer. This increased lateral repulsion could explain the decrease in plateau value with increasing pH value.

5.3.4 Ionic strength dependence and the influence of Ca^{2+} ions

In Figures 5.8 and 5.9 the influence of ionic strength is shown under electrostatically repulsive conditions using negatively charged PS-latex (B) at pH=8 and 10 respectively. The adsorption isotherm became higher affinity with increasing ionic strength (Figure 5.8). This confirmed the above drawn conclusions that electrostatic interactions are an important driving force for Lipolase adsorption. Increasing the ionic strength caused a decrease of repulsive electrostatic interactions between enzyme and surface and between adsorbed enzymes. This could explain the somewhat increased plateau values at higher ionic strength (Figure 5.9). In Figure 5.10 the ionic strength dependence is shown under electrostatically attractive conditions. Increasing the ionic strength caused only a small decrease in adsorption. This indicates that the attractive interaction between surface and enzyme is very strong at low ionic strength.

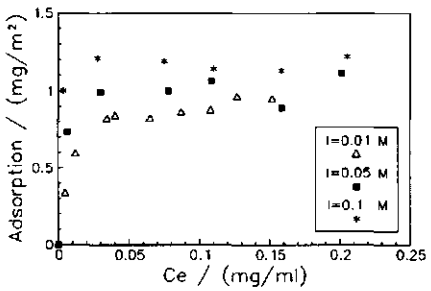

Figure 5.8 Lipolase adsorption isotherms at different ionic strengths on PS-latex B. Conditions: pH=8, T=30°C.

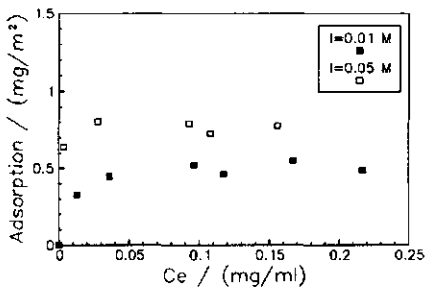

Figure 5.9 Lipolase adsorption isotherms at different ionic strengths on PS-latex B. Conditions: pH=10, T=30°C, low [Borax].

The importance of Ca^{2+} ions for lipolytic activity on hydrolysable interfaces is well known in the literature [20,21,22,23]. Addition of calcium ions results in an increased activity of the lipases such as pancreatic lipase, lipoprotein lipase and phospholipases. Several explanations were postulated over the years [24] of which four are briefly mentioned here. The calcium ions could decrease the concentration of free fatty acids therewith reducing the possible "product inhibition" of the lipase. The second possible mechanism is the adsorption of Ca^{2+} ions onto the oil-water interface compensating for the negative surface charge (and possibly also altering the surface

tension) caused by the presence of dissociated fatty acids. This decreases the repulsive electrostatic interaction between the negatively charged lipase and the interface [25] therewith increasing the enzyme adsorption. The third one is the interaction of calcium ions with the lipase molecule either decreasing its negative charge or changing its stability and making it a better adsorptive [26,27]. The fourth suggested explanation is the break-down of a high viscosity layer consisting of fatty acid-soaps at the interface which prevents obstruction of the enzyme in reaching the substrate [28]. The complexity of the hydrolysable interface system, induced by the lipolytic action, hampers the determination of the importance of these probable causes of this so-called calcium effect. We tried to investigate this Ca^{2+}-effect by examination of the influence of calcium ions on the adsorption on a non-hydrolysable surface.

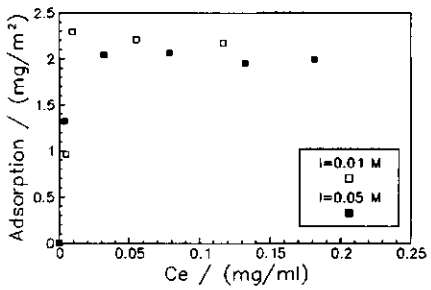

Figure 5.10 Lipolase adsorption isotherms at different ionic strengths on PS-latex C. Conditions: pH=8, T=30°C, low [Borax].

The adsorption isotherms at pH=8 and pH=10, measured from solutions with identical ionic strength that contained NaCl and $CaCl_2$, are shown in Figures 5.11 and 5.12 respectively. It is clear that even in this simple system, in the absence of free fatty acids and other hydrolysis products, the plateau value of the isotherm is increased by a factor of 2 in the presence of $CaCl_2$ at both pH values. We tried to distinguish between the interaction of calcium ions with the latex surface and the Lipolase molecule.

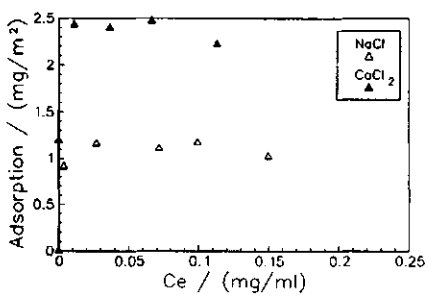

Figure 5.11 Ca^{2+} dependence of Lipolase adsorption isotherms on PS-latex B. Conditions: pH=8, I=0.05 M, T=30°C.

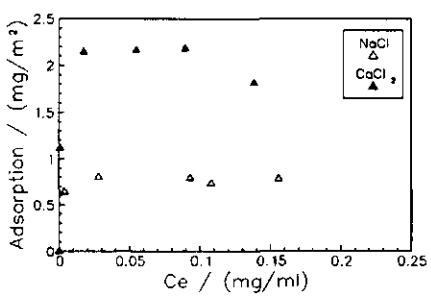

Figure 5.12 Ca^{2+} dependence of Lipolase adsorption isotherms on PS-latex B. Conditions: pH=10, I=0.05 M, T=30°C.

Capillary Zone Electrophoresis (CZE) on Lipolase in the presence and the absence of $CaCl_2$ was meant to determine the net charge on the enzyme. However, the experiments

failed to give interpretable electrographs when Ca^{2+} ions were present in the buffer solution. This was probably caused by precipitation of Lipolase on the silica capillary wall.

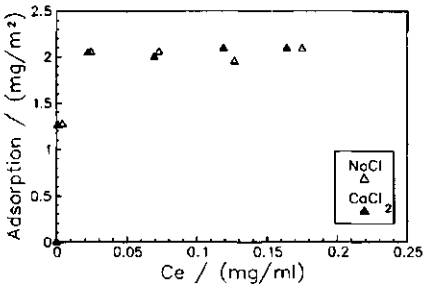

Figure 5.13 Ca^{2+} dependence of Lipolase adsorption isotherms on PS-latex C. Conditions: pH=8, I=0.05 M, T=30°C.

An alternative way to examine the interaction between Lipolase and Ca^{2+} was an adsorption experiment on positively charged latex C. If the uptake of one or more Ca^{2+} ions decreased the negative charge on the Lipolase significantly the adsorption on latex C would decrease. Figure 5.13 shows that the adsorption did not alter significantly upon addition of calcium ions. From this result we can not exclude the possibility that calcium ions associate with Lipolase as the above-reported absence of a decrease in adsorption upon the increase of ionic strength shows that the affinity for the surface is very strong under these experimental conditions.

On the other hand, from zeta-potential measurements of latex B we can conclude that Ca^{2+} ions adsorb onto the negatively charged latex. The measured zeta-potentials were in the absence and the presence of Ca^{2+} at pH=8: −47 and −12 mV, and at pH=10: −44 and −21 mV respectively (see also Table 3.4). The large decrease in zeta-potential and therefore in electrostatic repulsion between the enzyme and the surface can explain the sharp increase in adsorption when calcium is present. An alternative explanation for the increased adsorption could be that the calcium ions "bridge" between the enzyme and the surface. As discussed above, we can not yet exclude the contribution of an interaction between calcium ions and Lipolase. The mechanism by which calcium ions influence adsorption could be examined further by measuring the adsorption in presence of magnesium ions similar to the approach in the case of Savinase (section 3.3.6).

5.3.5 Temperature variation

The temperature dependence of Lipolase adsorption was examined under electrostatically attractive and repulsive conditions. In Figures 5.14 and 5.15 the adsorption isotherms are shown at 3°C and 30°C on negatively and positively charged latex respectively. Under repulsive conditions the adsorption increases with temperature. According to Le Chatelier's rule the increase in affinity with increasing temperature indicated that the adsorption was endothermic (a positive change in enthalpy) as expected for

electrostatically repulsive conditions. The adsorption occurred spontaneously and this meant that an increase of entropy (e.g. caused by dehydration of hydrophobic interfaces) must be the driving force for the adsorption under these conditions. Under electrostatically attractive conditions the plateau value of the adsorption isotherm is independent of temperature.

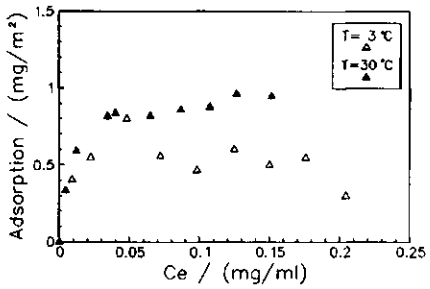

Figure 5.14 Lipolase adsorption isotherm at different temperatures on PS-latex B. Conditions: pH=8, I=0.01 M.

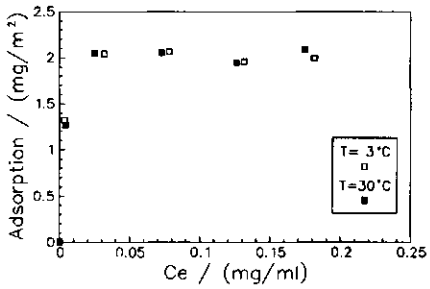

Figure 5.15 Lipolase adsorption isotherm at different temperatures on PS-latex C. Conditions: pH=8, I=0.05 M, low [Borax].

5.3.6 Adsorption measurement with the Radio-active Tracer Technique

The purity of "old" labelled [^{3}H]-Lipolase (batch 11-'88) was checked with Reversed Phase HPLC. The radio-activity in the collected one-minute-samples after HPLC separation is shown in Figure 5.16a and the cumulation of detected radio-activity as a function of the retention time is given in Figure 5.16b. Comparison of Figure 5.16a with the HPLC chromatogram of unlabelled Lipolase (Figure 5.19) clearly points to large differences between the labelled and unlabelled Lipolase.

The "new" procedure, developed for the labelling of Savinase was also used for the labelling of Lipolase. The HPLC chromatogram of the "new" [^{3}H]-Lipolase (batch 3-'91) is shown in Figure 5.17. It is clear that this material differed significantly from both the unlabelled and the "old" labelled material. This new batch consisted of protein that was more hydrophobic than the unlabelled Lipolase (compare with Figure 5.19). Consequence of this inequality is that the RTT method can not be used because the more hydrophobic material would adsorb preferentially on hydrophobic surfaces therewith giving erroneously high adsorption values. We conclude from these results that the labelling procedure that was used extensively in the literature for several proteins caused a degradation of Lipolase just as it caused degradation of Savinase and PMS-Savinase.

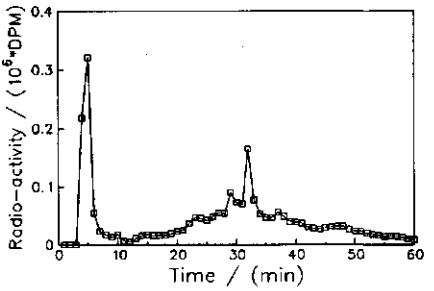

Figure 5.16a HPLC chromatogram of "old" batch 11-'88 [^{3}H]-Lipolase with detection of ^{3}H by collection of one minute fractions.

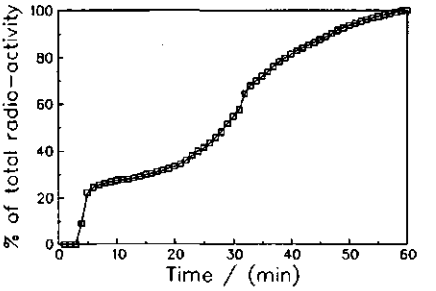

Figure 5.16b Cumulation of detected radio-activity after HPLC separation of [^{3}H]-Lipolase (batch 11-'88).

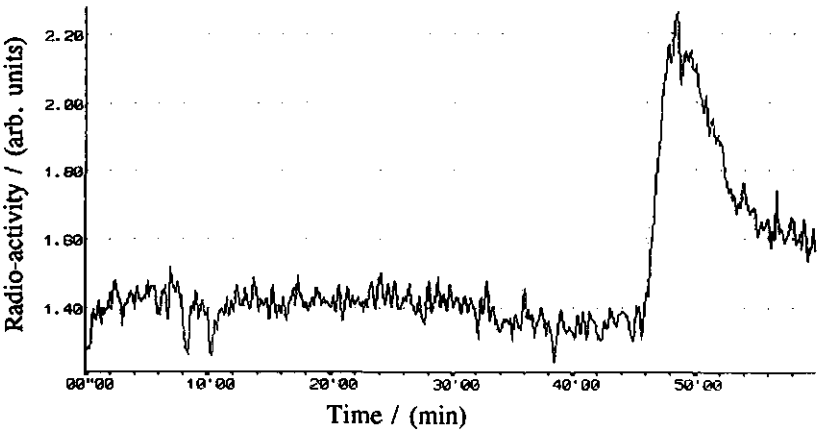

Figure 5.17 HPLC chromatogram of "new" batch 3'-91 [^{3}H]-Lipolase immediately after labelling with radio-activity detection.

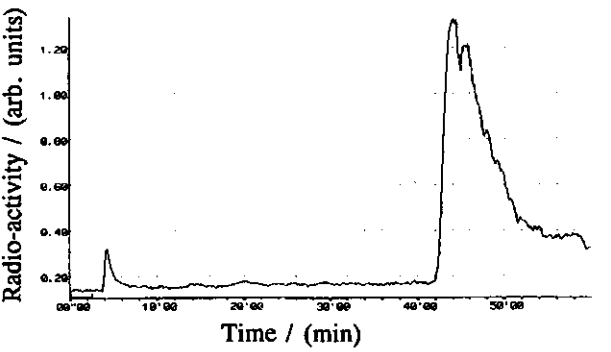

Figure 5.18 HPLC chromatogram of "new" batch 3-'91 [^{3}H]-Lipolase after 3 months storage at −20°C with radio-activity detection.

The labelling of proteases was in a way expected to be problematic because of the possibility of autolysis during the labelling reaction. We do not know why the labelling reaction (performed under very moderate conditions) caused such significant alterations of properties of Lipolase.

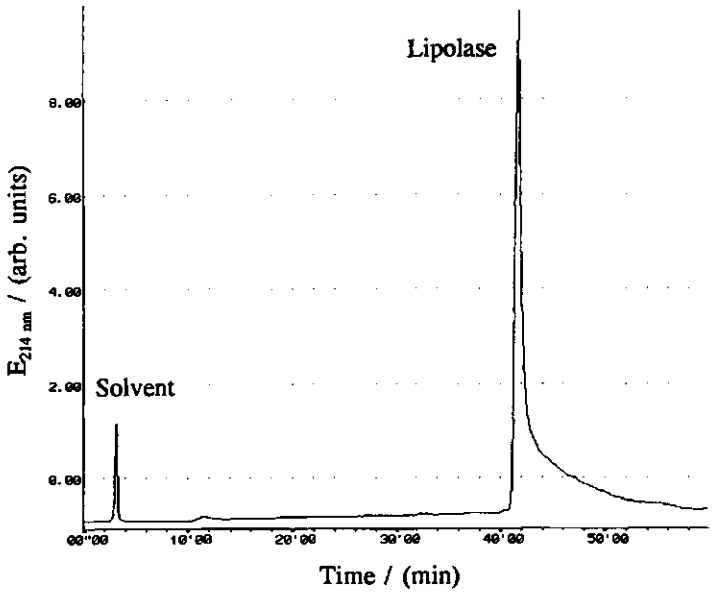

Figure 5.19 HPLC chromatogram of unlabelled Lipolase with UV 214 nm detection.

The storage stability of the newly labelled [^{3}H]-Lipolase was studied by analysis of the HPLC chromatograms after 3 months storage at $-20°C$ as shown in Figure 5.18. The tritium labelled enzyme already showed a peak at a very short retention time similar to the one detected in the "old" labelled enzyme. The radio-active material with very short retention time could be Lipolase fragments due to a very small amount of protease present in the Lipolase sample. Another possibility is that the tritium of the labelled methyl group, that is coupled to the lysine, is exchanged by a hydrogen atom from water molecules of the surrounding medium during storage.

The inequality of the labelled and unlabelled enzyme together with the rapid degradation of [^{3}H]-Lipolase leads to the conclusion that the RTT method is not suitable for Lipolase adsorption measurements.

5.4 Discussion

Taking into account the differences in isoelectric points of Savinase (pH=10) and Lipolase (pH=4.3) the similarity in adsorption behaviour is striking. In contrast to most proteins examined in the literature [29] these enzymes do not adsorb strongly on hydrophobic interfaces under electrostatically repulsive conditions. It is concluded that adsorption of these enzymes does not cause dehydration of the complete surface area of the adsorbent (see also Figure 3.29). If such limited dehydration were the case, the resulting loss of Gibbs free energy would dominate all other interactions and the enzyme would adsorb on any hydrophobic interface irrespective of the electrostatic interaction. The small dehydrated surface area indicates that these enzymes do not unfold strongly upon adsorption. This characteristic of Savinase and Lipolase could be related to the fact that these proteins are *enzymes* which need a specific conformation to retain their activity.

Comparing Savinase and Lipolase adsorption in more detail we see that the adsorption of negatively charged Lipolase on a negatively charged hydrophobic surface is higher than that of positively charged Savinase on a positively charged hydrophobic surface (Figure 3.6) while the electrostatic repulsion is smaller in the latter case. It could indicate that the dehydrated area on the adsorbent per enzyme molecule that adsorbs is larger for Lipolase than for Savinase. One could speculate that this, in turn, is caused by structural rearrangements in the Lipolase molecule e.g. the opening of the lid that covers the active site in solution. The negligible adsorption of Lipolase on glass shows either that the gain in entropy by this localized unfolding is not large enough to overcome the electrostatic repulsion or that this unfolding takes only place at a hydrophobic interface.

We conclude that the following interactions are of importance for Lipolase adsorption (see also section 1.3). The dehydration of the interface, possibly "assisted" by small structural rearrangements in the enzyme, is important and it enables the enzyme to adsorb selectively on hydrophobic interfaces. This is of crucial importance for the effectiveness of the lipase both under natural conditions and in fabric washing systems. The electrostatic interactions between the surface and the enzyme are very prominent due to the fact that the above-mentioned dehydration does not dominate the adsorption. Lateral repulsion between the adsorbed enzymes is also an important interaction which results in a plateau value of the adsorption isotherm that varies with e.g. pH and ionic strength. Just as it is the case for Savinase, Lipolase adsorption shows clearly that it is the *balance between the different types of interactions* that determines the adsorbed amount.

These adsorption experiments on non-hydrolysable solid interfaces give some insight into the adsorption mechanism that will be useful for understanding the working mechanism

of lipases on their natural substrate, triacylglycerol-water (liquid-liquid), interfaces. The importance of hydrophobic dehydration as the driving force for adsorption will direct the lipase towards the substrate. As lipolytic hydrolysis occurs the surface characteristics will change. Depending on the pH, the surface will become more negatively charged due to dissociation of fatty acids which are hydrolysis products. This increase in negative charge can decrease lipase adsorption and it can therefore function as a negative feed-back mechanism. Addition of calcium ions increased the adsorption on a negatively charged interface by a factor of two, even in the absence of hydrolysis products. It is therefore very likely that the decrease of negative surface charge by addition of Ca^{2+} ions together with a possible decrease in negative charge on the enzyme by association with the calcium ion causes larger adsorbed amounts on natural interfaces as well. However, as the enzymatic activity on an interface is reported to increase up to thousand-fold [9] the increased adsorption will not be the only effect of calcium addition. The delicate balance between the interactions determining Lipolase adsorption on negatively charged surfaces explains the general observation [3] that lipase adsorption is sensitive to the presence of other surface active components. As the affinity of Lipolase is not so strong it can easily be replaced by e.g. surfactants or other proteins.

5.5 Conclusions

Electrostatic interaction and dehydration of hydrophobic surfaces are the main factors that determine Lipolase adsorption. Under attractive electrostatic interaction between the surface and the enzyme the plateau value of the isotherm corresponds to a monolayer coverage (2.3 mg m^{-2}). Under experimental conditions in which dehydration of the hydrophobic surface is almost compensated by electrostatic repulsion between the enzyme and the sorbent, the lateral repulsion between the adsorbed enzymes determines the surface coverage of Lipolase. The adsorption mechanism of Lipolase is comparable with that of Savinase. The difference between Lipolase adsorption and that of proteins examined in the literature is that Lipolase adsorbs much less on hydrophobic interfaces. It is therefore concluded that the dehydrated surface area of the adsorbent is relatively small and consequently that the enzyme will not unfold significantly upon adsorption.

The implication for the adsorption on natural substrate interfaces is that the adsorption of negatively charged Lipolase on negatively charged surfaces will be low due to repulsive electrostatic interaction. The low affinity of the Lipolase molecule for the surface will also cause a strong decrease of adsorption upon addition of surface active components such as surfactants. The formation of (dissociated) fatty acids at the oil-water interface by lipolytic hydrolysis will be expected to increase the negative surface charge and therefore further decrease the adsorption. The adsorption on negatively charged latices

is increased by a factor two in the presence of Ca^{2+} ions. The calcium ions decrease the repulsive electrostatic interactions between the enzyme and the surface and therewith increase the adsorption. Under electrostatically repulsive conditions the adsorption increases with increasing temperature.

Both batches of radio-active labelled [^{3}H]-Lipolase examined here, were essentially different from the unlabelled enzyme. Therefore the reductive methylation procedure can not be used for radio-active labelling of Lipolase. Consequently the Radio-active Tracer Technique could not be used to determine Lipolase adsorption and the concentration determination was performed by Lowry colouring method.

5.6 References

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CHAPTER 6

SAVINASE™ ADSORPTION ON POLYESTER AND COTTON CLOTH

Abstract

In fabric washing detergent systems Savinase has to adsorb onto soiled cloth in order to remove protein containing stains. In this study the adsorption of Savinase on various textiles, such as polyester, cotton and cotton artificially soiled with the protein BSA (Bovine Serum Albumin) was examined. Enzyme adsorption on these practical solid surfaces proved to be much more complex than on the non-porous, non-hydrolysable, polystyrene latices and glass that were examined in Chapter 3.

The specific surface area of polyester was determined to be 0.27 $m^2 g^{-1}$ by adsorption of the protein lysozyme. Lysozyme adsorption on polyester reached equilibrium within 1 hour. The maximum adsorption of Savinase on polyester, attained under electrostatically attractive conditions, corresponded to monolayer coverage. The affinity of Savinase for the polyester surface was governed by electrostatic interaction together with dehydration of the hydrophobic interface.

The surface areas of clean and BSA-soiled cotton were determined to be 15 $m^2 g^{-1}$ and 9 $m^2 g^{-1}$ respectively by lysozyme adsorption measurement. However, the actual values could differ by 50% from these as lysozyme adsorption proved to depend on the nature of the interface. The steady state in adsorption on cotton was only reached after 8 hours contact time due to diffusion of protein into the hollow cotton fibres. The adsorption of Savinase on clean cotton resembled that on glass and is mainly governed by electrostatic interaction between the enzyme and the surface.

The adsorption values of active and PMSF-inhibited Savinase on cotton artificially soiled with BSA, were very different even after short contact times. Although further experimental evidence is necessary we provisionally conclude that the lower adsorption on BSA-soiled cotton of active Savinase compared to that of PMSF-inhibited Savinase was (partially) caused by the decreased affinity of the enzyme for the altered interface. Alteration of the solution by an increased concentration of hydrolysed BSA fragments did not contribute significantly to the decrease in adsorption.

6.1 Introduction

Savinase, an alkaline protease, is added to fabric washing detergent formulations to improve the removal of protein containing stains. In a washing machine the protease has to dissolve in the water, and subsequently adsorb on the soiled cloth, before it can hydrolyse the immobilised protein soil. In previous chapters we examined the adsorption of Savinase and Savinase variants on surfaces such as polystyrene latices and glass to establish the interactions involved in the adsorption process. In this chapter we will use these results to study the adsorption on surfaces which are important in practice such as polyester, clean cotton and protein-soiled cotton cloth.

Before we can compare the results with previously reported adsorption values on non-porous surfaces, the specific surface area of polyester and cotton cloth has to be determined. A method often used is the one of Brunauer, Emmett and Teller (B.E.T.) [1] which is based on N_2 adsorption on porous material. This method is known to determine only the outer surface of for example cotton fibres, resulting in much too small surface areas [2]. The collapse of the porous structure due to rigorous drying of the cotton and the low pressure during gas adsorption are considered to cause the low surface area. Therefore other methods are reported in literature such as dye adsorption measurement from solution and negative co-ion adsorption [3]. The reported surface areas measured using this type of experiments are of the order of 100 $m^2 g^{-1}$ but differ considerably from batch to batch as cotton is a natural fibre [3,4]. However, as protein molecules are quite large molecules it is expected that the effective surface area is smaller than established by dye- or ion-adsorption as proteins are unable to penetrate into the smaller pores.

Therefore, we determined the surface area in an alternative way. The adsorption of lysozyme on polyester and cotton cloth was measured and compared to the isotherms measured at non-porous interfaces. As it was concluded [5,6] that lysozyme adsorption is virtually independent of the surface characteristics, the specific surface area could be determined comparing the measured isotherms on polyester and cotton with those on glass and polystyrene latices. The dimensions of the lysozyme molecule are 4.5 x 3.0 x 3.0 nm^3 [5] and this is only slightly smaller than those of Savinase, which is a spherical molecule with a radius of 2.3 nm. Therefore, we assumed that the surface areas, accessible to Savinase and lysozyme, were identical.

As the enzyme's effectivity in a practical fabric washing system is not only governed by the steady-state adsorption value, we also determined the time dependence of Savinase adsorption. A typical wash lasts only about 30 minutes and that could be shorter than the

time it takes to reach adsorption equilibrium in a porous medium. In this study we are not only interested in the intrinsic adsorption of Savinase i.e. the adsorption without enzymatic hydrolysis but also in the influence of protein fragments released from the soiled cloth on the adsorption of the enzyme.

Primarily because of the complexity of the practical solid-liquid interfaces this chapter will be restricted to a first examination of the phenomena of importance in Savinase adsorption under fabric washing conditions. It is worth noting that although the system we examined here is already complex compared to the interfaces that are frequently used for protein adsorption studies, it is still very simple compared to the actual fabric washing system. For instance, in a washing machine the protease also has to compete for the surface with, for example, anionic and non-ionic surfactants while calcium binding components and bleach molecules influence both the enzyme and the surface characteristics. Therefore, the washing of clothes is an extremely complex process, and to try and understand it, let alone influence it, represents an enormous challenge.

6.2 Experimental

Materials

Both active and PMSF-inhibited Savinase were prepared according to the procedures reported in Chapter 2. Egg white lysozyme, lot number 39 F8213, was supplied by Sigma.

The negatively charged polystyrene latices A and B and the glass beads used in this study were described before in Chapter 3, section 3.2.1. Polyester cloth (HA 1996) was supplied by NK Hezemans Textiel B.V. (Waalre, The Netherlands) and cotton cloth by Phersee Kolbermoor (Augsburg, Germany). So-called BSA-soiled cotton was prepared by soiling prewashed cotton with chemically crosslinked BSA (Bovine Serum Albumin, ex Sigma, lot number 98F-0823), according to the procedure described in Appendix 6.1. Two batches of BSA-cotton were used in this study. The first one was stored for 18 months at 4 °C in the dark. The second batch was prepared just before use and stored under identical conditions.

In order to be able to determine small amounts of removed BSA, we labelled 10% of the BSA with the fluorescent label FITC (fluorescein isothiocyanate) supplied by Sigma. For casein the labelling procedure has been published before by Twining [7]. The coupling constant was calculated by dividing the concentration of FITC (using the molar extinction coefficient of FITC of 75,000 $l\,mol^{-1}\,cm^{-1}$ at 495 nm and pH=9) by the concentration of BSA based on the weighed mass of protein.

BSA removal measurement

The total amount of BSA removed from the cloth by hydrolysis was found by spectroscopic measurement of the concentration of FITC in the enzyme solution. FITC-BSA was detected at 495 nm and the pH dependency of the molar extinction coefficients of FITC-BSA is shown in Table 6.1. The molar extinction coefficient of FITC-BSA at pH=9 was assumed to be identical to the one for FITC.

Adsorption procedure

The adsorption was calculated from the depletion of enzyme from solution. The concentration of PMS-Savinase and the higher active Savinase concentrations were determined using the Lowry colouring method while lower Savinase concentrations were measured using the AAPF method. Both methods were described in detail in Chapter 2. The experiments were performed in 2 ml Eppendorf centrifuge tubes and the cloth was added as a number of small 5 x 7 mm^2 pieces. Savinase adsorption was determined at 3°C from the Borax buffer solutions indicated in Table 3.3. The adsorption of lysozyme was measured at pH=7 (0.01 M phosphate buffer) and 25°C.

Table 6.1 Relative molar extinction coefficients of FITC-BSA at various pH values.

pH	ϵ ($l\ mol^{-1}\ cm^{-1}$)	wavelength (nm)
8	0.67	493
9	1	495
10	1.12	496
11	1.10	495

6.3 Results and Discussion

6.3.1 Lysozyme adsorption on polystyrene latex and glass

In the literature [5,6], lysozyme adsorption is reported to be virtually independent of surface properties such as hydrophobicity and surface charge as shown in Figure 6.1. This characteristic is essential for the accurate determination of the surface area of polyester and cotton cloth. Comparison of the preliminary results on the adsorption on BSA-soiled and clean cotton shed doubts on the surface independent adsorption of lysozyme. Therefore, the adsorption values of lysozyme on PS-latices and glass were examined under identical circumstances applying various experimental techniques. As shown in Figure 6.2 the lysozyme adsorption depended on the nature of the surface. The adsorption on glass was significantly lower than on PS-latex A. The adsorption on PS-latex was higher than reported before and also depended on the surface charge and the

ionic strength. At the moment we have no explanation the differences between our experiments and those reported in the literature.

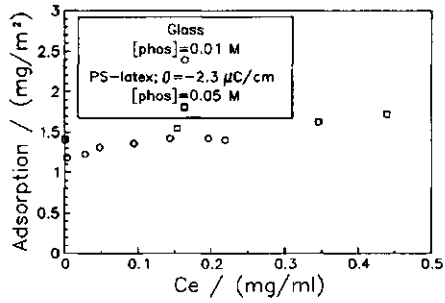


Figure 6.1 Literature values of lysozyme adsorption on PS-latex [5] ([phosphate]=0.05 M) and glass [6] ([phos]=0.01 M) as determined with UV 280 nm absorption.

The adsorption-dependence on the surface characteristics obstructed an adequate determination of the surface areas of the various cloths. Thus both the calculation of the calculation of the absolute adsorption values, and the comparison between the adsorption on the various cloths such as clean polyester and cotton and the adsorption on clean and soiled cotton can only be approximated. It will be necessary to use another molecule or particle of similar size to Savinase that adsorbs in a truly surface independent manner to determine the surface area. When the surface area is known, lysozyme adsorption could be used to probe the relative hydrophobicity of the surface.

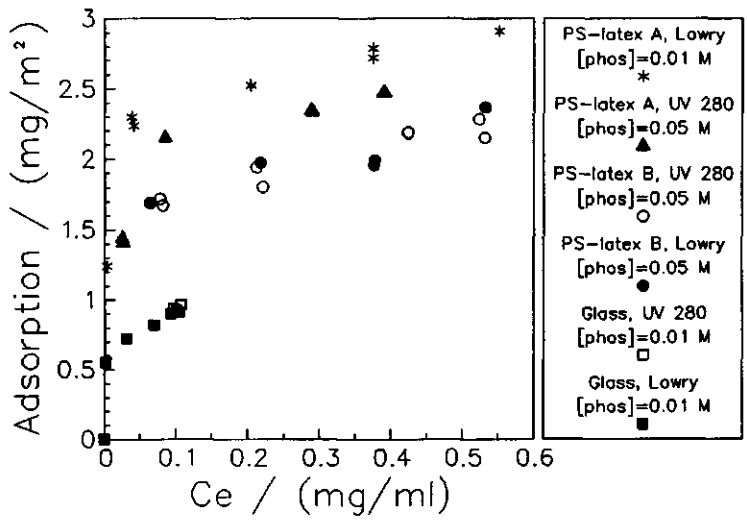


Figure 6.2 Adsorption of lysozyme on PS-latices A and B and on glass determined with various methods. The concentration of phosphate buffer is indicated in the legend of the figure.

6.3.2 Surface area determination of polyester and cotton

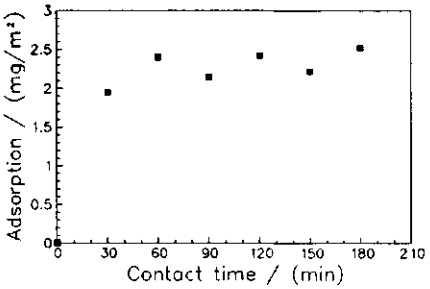
Polyester cloth

The specific surface area of polyester cloth can be estimated in a number of ways. A simple approach is to determine the fibre diameter with light microscopy and to calculate the surface area per gram of polyester, assuming all fibres to have an identical diameter and assuming a density of, say, 1000 kg m^{-3} for polyester. In this way the porosity of the fibres is neglected but the electron microscope picture in Figure 6.3 showed that this is not expected to lead to a large error in the surface determination. The measured diameter was 12 μm and the specific surface area calculated in this way was 0.3 $m^2 g^{-1}$. Another method frequently used is the B.E.T. nitrogen gas adsorption [1,2]. This method did not give a correct surface area as could be concluded from the fact that the surface area determined after long degassing of the cloth (6 hours) was 0.16 $m^2 g^{-1}$, which was less than the 0.27 $m^2 g^{-1}$ after degassing for only 1.5 hours. This decrease in specific surface area was caused by the "closing" of the smaller pores as could also be concluded from the difference in pore size distribution. Therefore, the B.E.T. surface area determination gave only a lower limit for the specific surface area just as reported for cotton fibres [2].

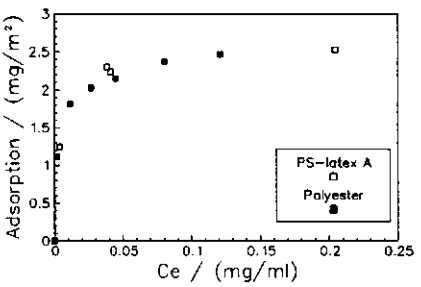
Figure 6.3 Electron microscope picture of a polyester fibre.

A third method, that is employed here, is the determination the surface area of polyester through the adsorption of lysozyme from solution. The contact time needed to reach a steady-state is 1 hour as can be seen in Figure 6.4 where the time dependence of lysozyme adsorption on polyester cloth is plotted.

The adsorption isotherm after 1 hour contact time is shown in Figure 6.5. A full match of the adsorption values on PS-latex A and polyester was obtained when a specific surface area of 0.27 $m^2 g^{-1}$ was used for polyester cloth. This value is in accordance with the above calculated 0.3 $m^2 g^{-1}$ based on the radius of the fibre. It suggests that polyester is a hydrophobic surface which is in line with expectation. In the following calculations we will use a specific surface area of 0.27 $m^2 g^{-1}$ for polyester cloth.



Figuur 6.4 Lysozyme adsorption on polyester as a function of time. Conditions: C_e=0.12 mg/ml, S=0.27 $m^2 g^{-1}$.



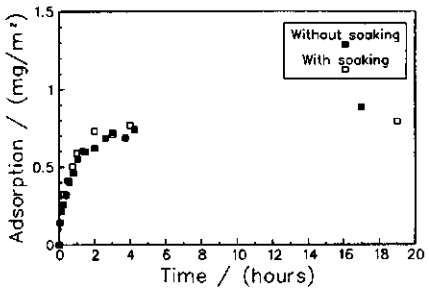
Figuur 6.5 Lysozyme adsorption isotherm on polyester and PS-latex A. Conditions: t_c=1 hour, S=0.27 $m^2 g^{-1}$.

Cotton cloth

The time dependence of lysozyme adsorption on cotton cloth (both clean and BSA-soiled) differed strongly from the one on polyester cloth. It took about 8 hours to reach a steady-state (Figures 6.6 and 6.7). The slow attainment of equilibrium could be explained by the porosity of the material, as shown in the electron microscope photograph of a cotton fibre (Figure 6.8). The time necessary for Savinase to diffuse through the lumen of the cotton fibres (the main cavity in the cotton fibre, see Figure 6.8) corresponds to a realistic length of the lumen in the fibres of about 1.5 mm (with a diffusion coefficient of 9.5 10^{-11} $m^2 sec^{-1}$).

It was verified that the adsorption was not retarded by slow dissolution of gas, trapped in the small pores in the fibres. Figure 6.6 shows that the time dependence of lysozyme adsorption on cotton that is soaked in the buffer solution for 18 hours is not significantly different from that on "dry" cotton.

In order to determine the absolute value of the adsorbed amount per surface area the specific surface area must be determined. As discussed in section 6.3.1 an assumption must be made with respect to the nature of the surface of the cloth in order to compare the isotherms with those measured on non-porous materials. For the present purpose we will assume that clean cotton resembles glass while BSA-soiled cotton resembles the surface characteristics of PS-latex A.



Figuur 6.6 Lysozyme adsorption on clean cotton with and without soaking. Conditions: $S=15\ m^2\,g^{-1}$, soaking time 18 hours.

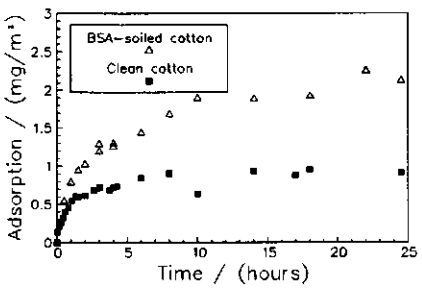
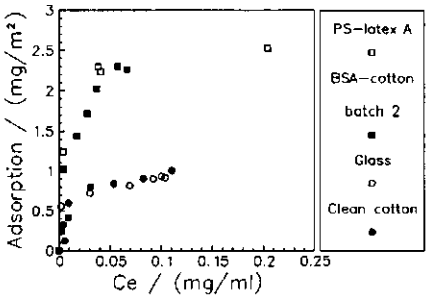


Figure 6.7 Lysozyme adsorption on BSA-soiled and clean cotton (batch 2) without soaking. $S_{BSA}=9\ m^2\,g^{-1}$, $S_{clean}=15\ m^2\,g^{-1}$.

Figure 6.8 Electron microscope photograph of a cotton fibre.

In order to understand the reason for this choice the other obvious possibility must be considered namely that the affinity of lysozyme for both surfaces would be identical and comparable to the adsorption values reported in the literature [5,6]. In Figures 6.9 and 6.10 the two alternatives are shown. In Figure 6.9 the isotherms are superimposed on the ones, measured by us, on glass and PS-latex A (see also Figure 6.2). The specific surface areas determined in this way are 15 $m^2\,g^{-1}$ for clean cotton and 9 $m^2\,g^{-1}$ for BSA-cotton. However, in Figure 6.10 the isotherms on clean and BSA-soiled cotton are

superimposed on each other and shifted to the same absolute level as the lysozyme adsorption reported in the literature (see also Figure 6.1). The calculated specific surface areas are 8.5 $m^2 g^{-1}$ for clean cotton and 15 $m^2 g^{-1}$ for BSA-soiled cotton.



Figuur 6.9 Comparison of lysozyme adsorption isotherms on cotton (t_c=17 hours) with isotherms on glass and PS-latex.

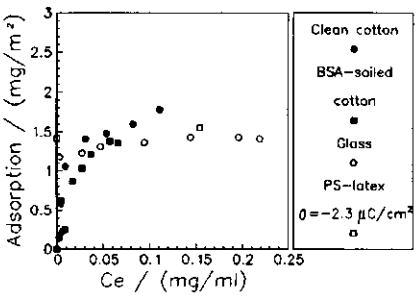


Figure 6.10 Comparison of lysozyme on clean and BSA-soiled cotton with literature values.

The latter comparison led to a higher specific surface area for BSA-soiled cotton than for clean cotton and this is very unlikely. During the soiling, the high concentration BSA-solution is taken up by the cotton and as BSA adsorbs at the cotton-water interfaces it is expected to fill the small pores. Therefore, one would expect the surface area of BSA-soiled cotton to be smaller than that of clean cotton. This view of the way cotton is soiled is based on two elements. The first is that BSA covers cotton roughly as a single layer. The total amount of soil per surface area of 3% w/w indicating that the adsorbed amount did not exceed the order of a monolayer coverage of about 2 mg m^{-2} [8]. The second assumption is that a part of the pores in the cotton are initially smaller than the diameter of a BSA molecule (about 4 nm radius), and this could be supported by a rough estimate of the average pore size. Cotton cloth in contact with water was measured to take up water. The increase in mass of the cloth is about 100%. From this it could be deduced that 1 g of cotton has a water-accessible volume of 1 cm^3. Assuming the pores to be cylindrical, the volume to surface ratio can be written according to equation (1):

$$\text{average pore radius} = 2 \cdot \text{volume} / \text{surface area} \qquad (1)$$

Assuming the water-accessible surface area to be about 100 $m^2 g^{-1}$ [3,9] the average pore radius is 20 nm. As the average pore size is only 2.5 times as large as the diameter of the adsorbing BSA molecule it is not unrealistic to assume that the small pores of the cotton are blocked by the soiling and not accessible for the adsorption of proteins such as lysozyme and Savinase. This results in a lower specific surface area of BSA-soiled cotton compared to that of clean cotton. Therefore the surface area determination based on the comparison shown in Figure 6.10, can not be correct. We conclude that the

affinities of lysozyme for clean and BSA-soiled cotton are different in a similar way as the affinities for glass and PS-latex (see also in Figure 6.2).

Although the surface characteristics such as charge and hydrophobicity of BSA-cotton and PS-latex A are probably not identical, the surface areas will be used that were obtained by superimposing BSA-cotton on PS-latex A. This could cause an inaccuracy in the surface area and thus lead to systematic errors in the determined adsorption values. Therefore, care must be taken comparing the absolute adsorption levels of Savinase at the various interfaces.

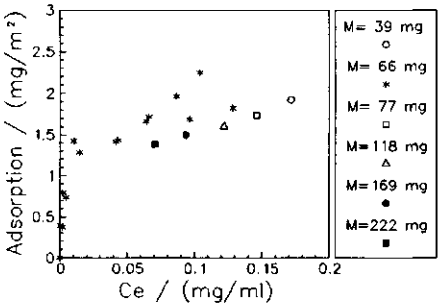


Figure 6.11 Surface area dependence (indicated by added mass) of lysozyme adsorption on BSA-cotton (batch 1) with t_c=21 hours.

The surface area dependency and the reproducibility of the adsorption on cotton cloth were examined using an old batch (number 1) of BSA-soiled cotton; the specific surface area of that batch was determined to be 6.5 $m^2 g^{-1}$. Even though no significant release of BSA from the BSA-soiled cotton could be detected it was verified that the lysozyme adsorption was independent of the volume to surface ratio (Figure 6.11). The absence of this dependency is characteristic for adsorption from monodisperse solutions ([10], see also Chapter 2). The reproducibility of the adsorption on clean and BSA-soiled cotton is shown in Figures 6.12 and 6.13.

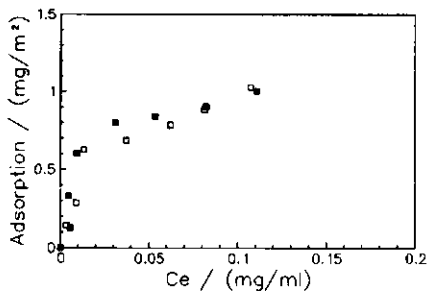


Figure 6.12 Reproducibility of lysozyme adsorption on clean cotton; S=15 $m^2 g^{-1}$.

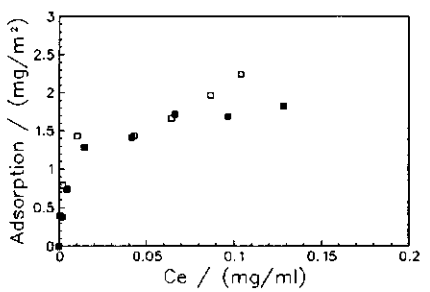


Figure 6.13 Reproducibility of lysozyme adsorption on BSA-soiled cotton (batch 1). S=6.5 $m^2 g^{-1}$.

6.3.3 Savinase adsorption on polyester cloth

The Savinase adsorption on polyester cloth was determined at pH=8 (Figure 6.14) and at pH=10 (Figure 6.15). Under attractive electrostatic conditions (pH=8) the adsorption is of the order of a monolayer coverage. At pH=10 there is still significant adsorption although there is hardly any electrostatic attraction, indicating that the polyester is hydrophobic. Dehydration of the polyester surface results in Savinase adsorption under these conditions just as it is the case with negatively charged PS-latices.

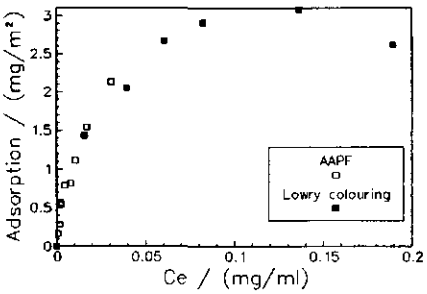


Figure 6.14 Savinase and PMS-Savinase adsorption on polyester cloth. Conditions: pH=8, I=0.01 M measured with AAPF and Lowry colouring method respectively.

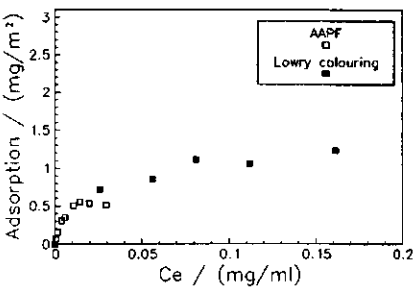


Figure 6.15 Savinase and PMS-Savinase adsorption on polyester cloth. Conditions: pH=10, I=0.01 M measured with AAPF and Lowry colouring method respectively.

When we compare the adsorption on polyester with that on polystyrene latex at an ionic strength of 0.01 M (Figure 3.1) we notice that the isotherms show a lower affinity (smaller slope at low concentration) and are somewhat more pH dependent. This could be caused by a lower surface charge at pH=8, possibly together with the fact that the polyester surface is titrated between pH=8 and pH=10. We could not confirm these explanations as the reproducibility of the surface charge determination on polyester by potentiometric titration was unsatisfactory, possibly due to hysteresis of the material at extreme pH values.

From these measurements it can be concluded that Savinase adsorbs in significant amounts on *clean* polyester at the pH and ionic strength that are normally used for fabric washing. Increasing the pH will decrease the adsorption due to a reduction of the electrostatic attraction between the enzyme and the surface.

6.3.4 Savinase adsorption on clean cotton cloth

Savinase adsorption reached equilibrium after more than 4 hours (Figure 6.16) just as was the case for lysozyme adsorption. The time necessary to reach equilibrium was

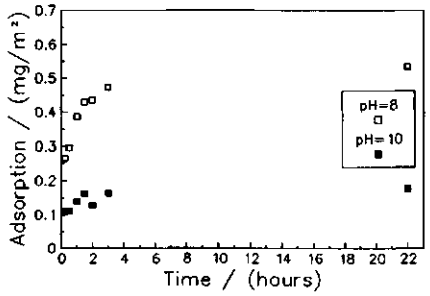


Figure 6.16 Time dependence of PMS-Savinase adsorption on clean cotton. Conditions: C_o=0.13 mg ml^{-1}, I=0.01 M.

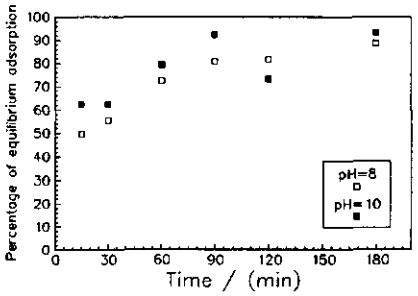


Figure 6.17 Time dependence of PMS-Savinase adsorption as a percentage of equilibrium value on clean cotton. Conditions: C_o=0.13 mg ml^{-1}, I=0.01 M.

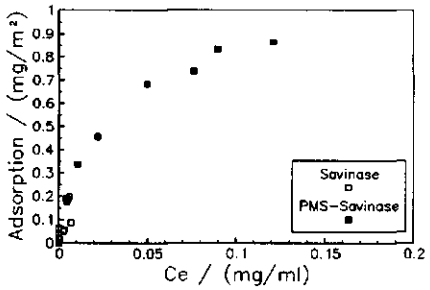


Figure 6.18 Corrected adsorption isotherm of Savinase ($t_{c,exp}$=2 hours) and PMS-Savinase ($t_{c,exp}$=4 hours) on clean cotton. Conditions: pH=8, I=0.01 M.

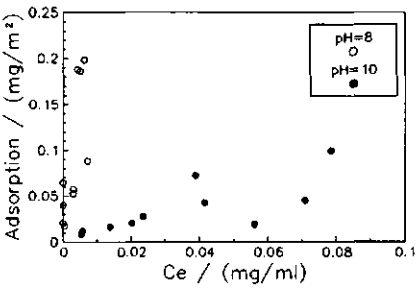


Figure 6.19 Corrected adsorption isotherms of Savinase on clean cotton at low concentration. Conditions: I=0.01 M, $t_{c,exp}$=2 hours.

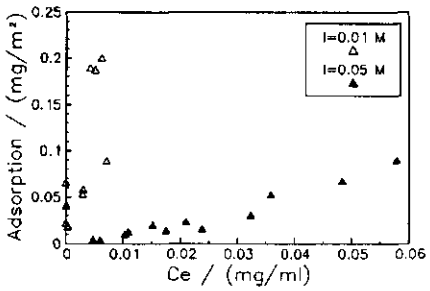


Figure 6.20 Corrected adsorption isotherms of Savinase on clean cotton. Conditions: pH=8, $t_{c,exp}$=2 hours.

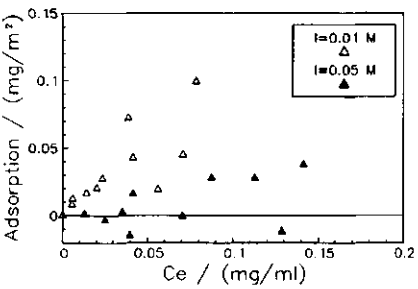


Figure 6.21 Corrected adsorption isotherms of Savinase on clean cotton. Conditions: pH=10, $t_{c,exp}$=2 hours.

independent of the affinity of Savinase for the surface, as can be concluded from Figure 6.17. From Figure 6.17 it can be concluded that after a washing time of 30 minutes 60% of the equilibrium value is reached. As at low temperature *active* Savinase degrades itself in solution (autolysis), it was experimentally impossible to determine its steady-state adsorption. Instead, we measured the adsorption after a relatively short contact time of 2 hours for active Savinase and 4 hours for PMS-Savinase and calculated the equilibrium values using the results shown in Figure 6.17. We assumed that after 2 hours contact time (indicated as $t_{c,exp}$) 80% of the equilibrium value was reached and after 4 hours 90%. The isotherms derived in this way are shown in Figures 6.18 to 6.21.

The plateau value of the isotherm under electrostatically attractive conditions was of the order of monolayer coverage (Figure 6.18). Just as it was the case for glass (Chapter 3) the pH dependence of Savinase adsorption on clean cotton was very strong (Figure 6.19). This indicated that dehydration of the interface was not very important. The importance of electrostatic interaction between the enzyme and the surface was evident from the strong decrease of adsorption with increasing ionic strength especially under attractive electrostatic conditions (Figures 6.20 and 6.21). These results indicated that the trends in the adsorption of Savinase on clean cotton are similar to those obtained from model surfaces such as glass. Therefore, the pH of the enzyme solution at low ionic strength should be higher than 10 to prevent strong adsorption of Savinase on *clean* cotton.

6.3.5 Savinase adsorption on BSA-soiled cotton

The adsorption of Savinase, and even of PMS-Savinase, on BSA-soiled cotton caused significant removal of BSA from the cotton at 3°C. The hydrolysis of proteins immobilised at the interface led to a strong reduction of the adsorption of the enzyme. This was concluded from the much lower adsorbed amount of active Savinase compared to that of PMS-Savinase (Figure 6.22a) together with the slight maximum in the adsorption of active Savinase as a function of time (Figure 6.22b). As the intrinsic adsorption of Savinase and PMS-Savinase on non-hydrolysable soil were identical (section 3.3.2), this difference was attributed to the effects of hydrolysis. The distinction between active and PMS-Savinase adsorption is even underestimated as the adsorption of PMS-Savinase was measured using the Lowry colouring method which detects all proteins, enzyme and removed BSA-fragments, in solution. Therefore the measured protein concentration exceeds the enzyme concentration and thus the calculated adsorbed amount will be too low. The adsorption values after longer contact times are thought not to be influenced by autolysis as protein fragments were shown to stabilise Savinase (Figure 2.32).

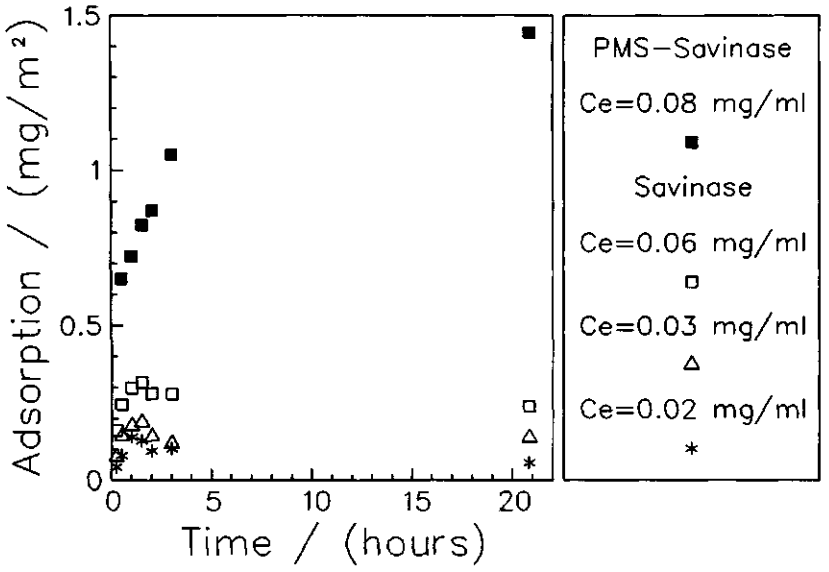


Figure 6.22a Time dependence of Savinase adsorption on BSA-soiled cotton. Conditions: pH=8, I=0.01 M.

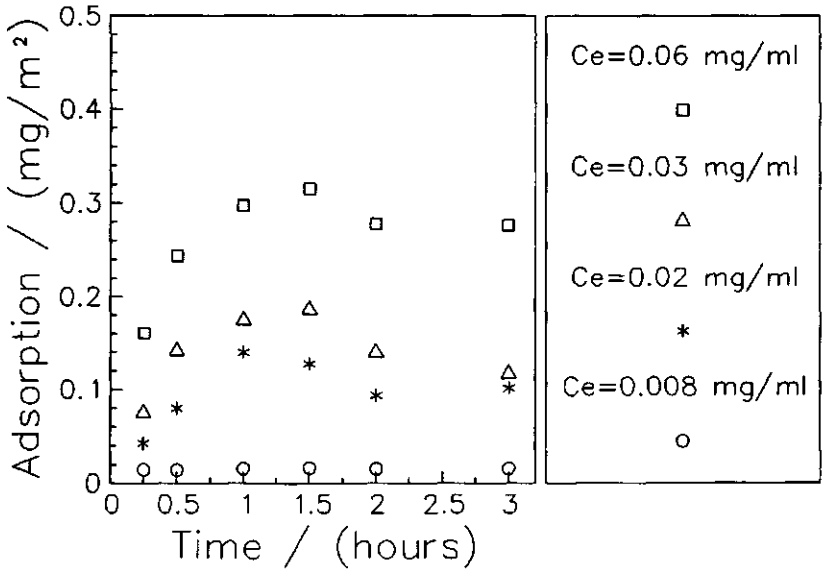


Figure 6.22b Time dependence of Savinase adsorption on BSA-soiled cotton. Identical to results in previous figure at short times.

A very interesting question is: how the enzymatic activity is influenced by the adsorption of Savinase. After contact with the enzyme both the surface and the solution can obtain different characteristics and both types of changes can alter the adsorption of Savinase. Hydrolysis of the soil-proteins increases the number of charged groups (both positively and negatively charged) and therewith probably the hydrophilicity of the surface. But after one or more "scissions" fragments of the soil-protein are not entangled any more in the soil matrix. They diffuse more easily from the interface and thereby alter the solution from which the enzyme has to adsorb. Another effect could be that the catalytic activity of the enzyme changed the *desorption* rate of Savinase. At a non-hydrolysable interface the enzyme did not desorb by dilution but by alteration of the adsorption energy e.g. changing the pH or ionic strength did lead to desorption, as shown in section 3.3.8. Hydrolysis of the immobilised protein on which Savinase is adsorbed could enhance desorption, as the complex of Savinase with the BSA-fragment is on the average more negatively charged than the enzyme itself (the isoelectric point of BSA is at pH=5.3 [11]). In solution the complex of the enzyme with the fragment can dissociate as the fragment is hydrolysed further. These possible explanations for the strong difference between the adsorption values of active and inhibited Savinase, even after short contact times, were investigated further.

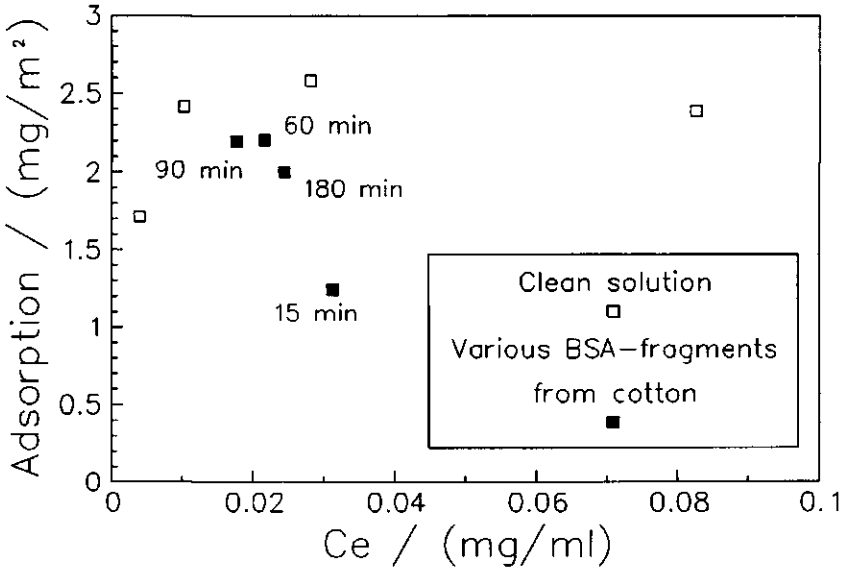


Figure 6.23 Savinase adsorption after exposure to BSA-soiled cotton, t_c indicated (closed symbols) and the adsorption on pure PS-latex A (open symbols, t_c=30 min).

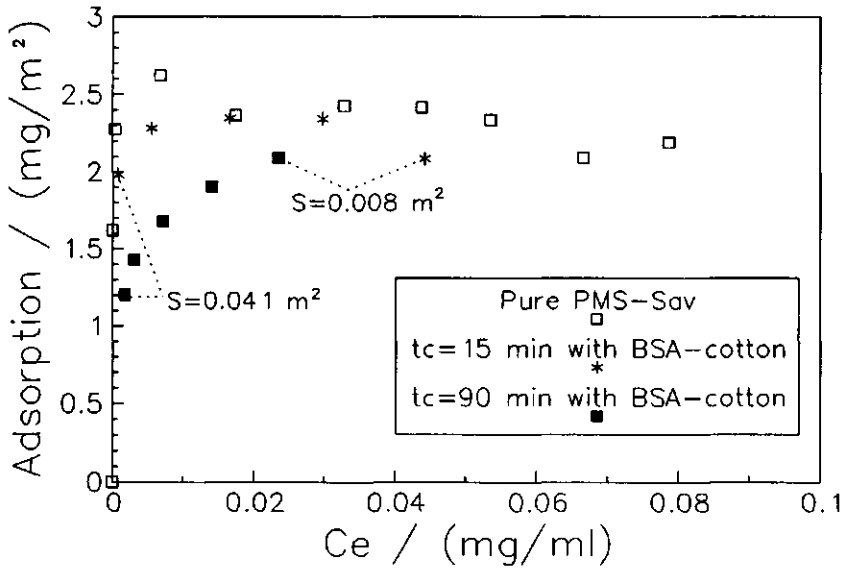


Figure 6.24 Savinase adsorption various surface areas (extreme values indicated in the figure) of PS-latex A after contact with BSA-soiled cotton. Conditions: pH=8, I=0.01 M.

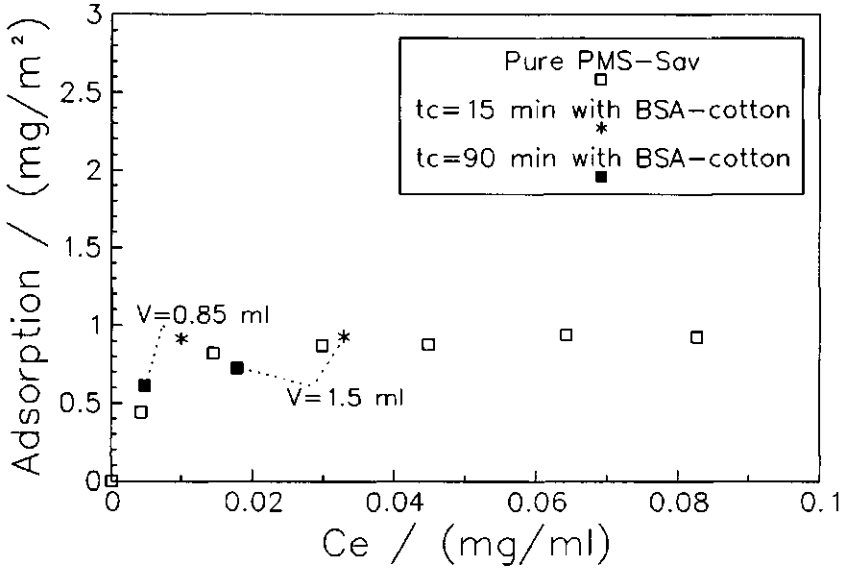


Figure 6.25 Savinase adsorption on glass from a solution previously in contact with BSA-cotton. Conditions: pH=8, I=0.01 M.

The influence of the alteration of *solution* properties on the adsorption of active Savinase was examined by studying the adsorption on well characterised solid, non-porous, surfaces such as PS-latex and glass from solutions that were first brought in contact with BSA-soiled cotton for a certain time. In Figure 6.23 the adsorption on PS-latex A is shown from these solutions are compared with the adsorption isotherm of Savinase from uncontaminated solutions at pH=8 and ionic strength 0.01 M (Chapter 3). Apart from one measurement after a short contact time, no significant differences are observed between the adsorption values from a pure and a contaminated solution. It is worthwhile noting that the adsorption from contaminated solutions was not reproducible (especially after short contact times with the BSA-soiled cotton, see Table 6.2). Because of this irreproducibility, the experiments were extended to include a variation of surface area of the added sorbent. In this way the probability of competition between Savinase and BSA-fragments for the surface could be examined further.

If competition occurs between proteins with different affinities for the surface, there are two possible effect on adsorption (Chapter 2). The first possibility is that Savinase has the highest affinity and therefore wins the competition. In that case Savinase adsorption should follow the same isotherm as from an uncontaminated solution. If there are proteins (BSA-fragments) present in solution with a higher affinity for the surface, decreasing the surface area would lead to desorption of the Savinase [10].

Table 6.2 Reproducibility of the adsorption of Savinase on PS-latex A from solutions that were in contact with BSA-soiled cotton.

t_c with BSA-soiled cotton (min)	C_e (mg ml^{-1})	Adsorption (mg m^{-2})
15	0.031	1.24
15	0.044	2.09
90	0.018	2.20
90	0.018	1.95

The surface area dependency was investigated for two situations: (i) after 15 minutes and (ii) after 90 minutes contact time with the BSA-soiled cotton. Figure 6.24 shows that the "15 minutes"-BSA-fragments did not influence the adsorption of Savinase. This indicates that these fragments did not "win" the competition with the Savinase for the PS-latex surface. However, the "90 minutes"-BSA-fragments caused a surface area dependence of the Savinase adsorption. Increasing the surface area decreased the adsorption more than could be expected from the decrease in Savinase concentration in solution (C_e). Although the adsorption was dependent on the surface area, it did not follow either one of the above indicated possibilities. This is a first indication that competition is not very important in the case of the adsorption of Savinase and BSA-fragments on PS-latex A.

As this result could depend on the nature of the interface a limited set of experiments was repeated on glass. Figure 6.25 shows that competition could not be proven to influence Savinase adsorption on glass. Although the influence of the solution characteristics is not yet completely understood and it can be concluded that the strong reduction in adsorption of active Savinase (Figure 6.22a) is unlikely to be caused by changes in solution properties.

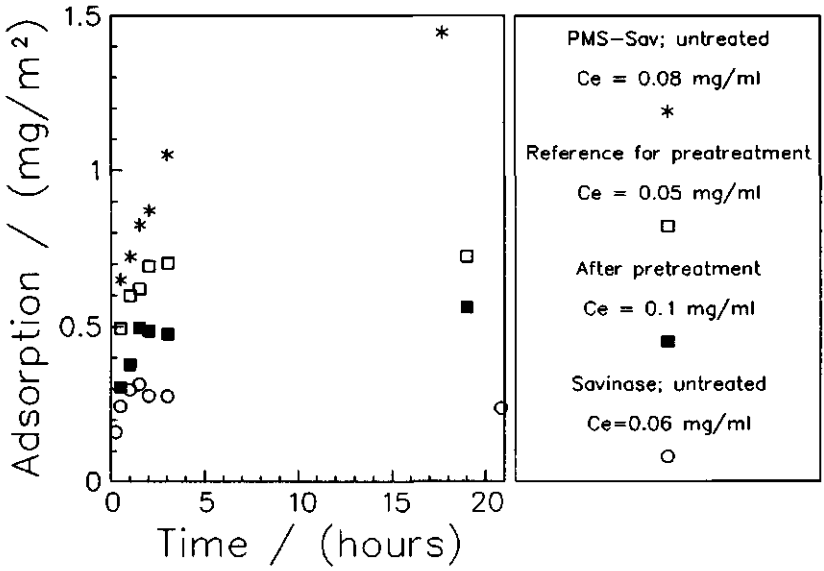


Figure 6.26 The adsorption of PMS-Savinase on pretreated and untreated BSA-soiled cotton and the comparison with active Savinase. Conditions: pH=8, I=0.01 M.

Therefore, another possible explanation for the strong reduction of the adsorption upon hydrolysis was examined: changes in the *properties of the BSA-soiled cotton* by the hydrolysis. We determined the adsorbed amount of PMS-Savinase on a BSA-soiled cotton that was pretreated with active Savinase. The pretreatment consisted of two steps. The first was to bring the BSA-cotton in contact for 1 hour with a solution of active Savinase leading to a concentration of 0.05 mg cm^{-3} and an adsorption of 0.3 mg m^{-2}. The second step was the desorption of 90% of the adsorbed Savinase and removal of hydrolysed BSA-fragments by rinsing the cloth three times for 30 minutes in a buffer solution (pH=10.5, ionic strength 0.1 M) and thereafter, rinsing in demi water. These conditions lead to desorption of the enzyme, as has been shown in section 3.3.8. After these pretreatment steps 65% of the initially present BSA was removed from the cotton surface.

The adsorption of PMS-Savinase on this pretreated BSA-soiled cotton is presented in Figure 6.26. It is compared with the adsorption on BSA-soiled cotton that only had gone through the second pretreatment step, that is the rinsing with buffer and demi-water. In Figure 6.26 the above-reported (Figure 6.22a) adsorption of PMS-Savinase and active Savinase on completely untreated BSA-soiled cotton are included for comparison. These results suggest that the characteristics of the interface were altered considerably by the hydrolysis and/or the removal of the immobilised protein. An accurate comparison of the decrease in adsorption by pretreatment with the difference in adsorbed amount of PMSF-inhibited and active Savinase is hindered by the fact that the C_e values varied. As the system is not at equilibrium (hydrolysis still occurred even with PMS-Savinase) the adsorbed amounts could not be corrected for these differences in concentration. We conclude provisionally that the lower adsorption on BSA-soiled cotton of active Savinase, as compared to that of PMSF-inhibited Savinase, was (partially) caused by the decreased affinity of the enzyme for the altered interface.

As stated before, the results obtained here are only a first step in examining the complex system consisting of active Savinase and protein-soiled surfaces. Because of the strong influence of protein fragments on Savinase adsorption, the determination of the *intrinsic* adsorption of Savinase onto protein-soiled cotton is not possible; so far we are unable to suppress hydrolysis completely. Adsorbing PMS-Savinase at relatively high concentrations (necessary for the detection with the Lowry colouring method) led to considerable removal and this made the detection method unreliable. As completely inactive Savinase was not available it could not be verified whether Savinase adsorbed preferentially on protein-soiled over clean cloth.

6.4 Conclusions

The specific surface area of polyester was determined to be 0.27 $m^2 g^{-1}$ by adsorption measurements of the protein lysozyme. Lysozyme adsorption on polyester attained equilibrium within 1 hour. Under electrostatically attractive conditions, the adsorption of Savinase on polyester was of the order of a monolayer coverage. The affinity of Savinase for the polyester surface was governed by electrostatic interaction together with dehydration of the hydrophobic interface.

The surface areas of clean and BSA-soiled cotton were determined to be 15 $m^2 g^{-1}$ and 9 $m^2 g^{-1}$ respectively using the adsorption of lysozyme. However, the actual values could differ by 50% as lysozyme adsorption depends on the nature of the interface. The surface area determination according to the B.E.T. method led to erroneously small surfaces as the porous structure collapsed at low pressure.

The steady state in adsorption on cotton was only reached after 8 hours contact time due to diffusion of protein in the hollow cotton fibres. The adsorption of Savinase on clean cotton resembled the adsorption on glass and is mainly determined by electrostatic interaction between the enzyme and the surface.

The adsorption of active and PMSF-inhibited Savinase on cotton artificially soiled with BSA differed very strongly even after short contact times. We conclude provisionally that the lower adsorption on BSA-soiled cotton of active Savinase compared to that of PMSF-inhibited Savinase was (partially) caused by the decreased affinity of the enzyme for the altered interface. Alteration of the solution by an increased concentration of hydrolysed BSA fragments did not contribute significantly to the decrease in adsorption.

6.5 References

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APPENDIX 6.1

PREPARATION OF BSA-SOILED COTTON

1. Prepare 1 litre of 4 w/w% BSA solution. 10% of the BSA added is labelled with the fluorescent dye FITC.

2. Just before starting the soiling procedure 1 ml of a 25% glutaraldehyde solution is added to 125 ml BSA / FITC-BSA solution and it is mixed for exactly 10 seconds with an Ultra Turrax. In order to obtain more strongly crosslinked BSA one can either stir the solution longer before going to step 3 or one can use a higher concentration of glutaraldehyde.

3. The soiling solution is brought onto the two rollers of a Benz walz soiling machine (type LHF 350). The pressure between the rollers is set at 40 kg cm^{-2} and revolving speed at 3.5 m min^{-1}.

4. Four pieces (10 x 50 cm^2 each) of clean cotton are soiled sequentially by the rollers of the soiling machine.

5. In order to repeat the procedure the used soiling solution must be discarded and one must start again with step 2 indicated above.

6. The soiled cloths are dried overnight at ambient temperature.

7. The testcloths are washed 3 times for 1 hour in a 0.01 M Borax pH=9 buffer in order to remove unlinked BSA.

8. The soil content of the testcloths can be determined by weighting of clean and soiled pieces immediately after the soiling (step 4) subtracting the protein washed from the cloth in step 7.

SUMMARY

ENZYME ADSORPTION AT SOLID-LIQUID INTERFACES

Enzymes are proteins with the capacity of catalysing various reactions. Nowadays two types of enzymes, proteases and lipases, are available for use in detergent formulations for household and industrial laundry washing. Proteases are capable of catalysing the hydrolysis of proteins while lipases enable the hydrolysis of glycerol esters, the main component in fats and non-mineral oils. In this study, two enzymes each representing one of these categories were examined: Savinase™, an extracellular serine protease of the subtilisin family from *Bacillus lentus* and Lipolase™, an extracellular lipase from the thermophilic fungus *Humicola Lanuginosa* S-38. To improve the soil- and stain removal in laundry washing the enzyme has to adsorb onto the soiled fabric and hydrolyse one or more of the stain components. This thesis describes a study on the mechanisms by which these enzymes adsorb at various solid-liquid interfaces from buffered solutions in the absence of other components that are found in common detergents.

Chapter 1 gives a general introduction. It contains an overview of the physico-chemical characteristics of the two enzymes and a summary of the present insight into the protein adsorption. In Chapter 2 the investigation of the adsorption measurement techniques for Savinase is reported together with methods used to inhibit the enzyme with the small organic inhibitor PMSF (phenylmethanesulfonyl fluoride) to suppress autolysis.

Several techniques are known to measure the protein concentration in solution and, hence, to determine the adsorption. However, the measurement of a *proteolytic* enzyme, such as Savinase, at such low concentrations as used in laundry washing systems proved to be a serious complicating factor. Proteolytic enzymes hydrolyses proteins and as the enzyme itself is a protein it is capable of self-destruction or cannibalism, so-called autolysis. Of the four well known methods for measurement of the protein concentration only two proved to give reliable results. The radio-active tracer technique could not be used for the measurement of Savinase adsorption because the unlabelled enzyme adsorbed preferentially over the labelled one. The latter was shown to be degraded by autolysis during the labelling reaction and subsequent storage. The competition between the numerous protein fragments resulted in a surface area- and volume-dependent adsorption comparable to the adsorption from polydisperse polymer solutions. The detection limit of the UV 280 nm extinction method was too low to determine the rising part of the adsorption isotherm. The Lowry colouring method gave reliable results but it can not be

used to discriminate Savinase in a protein mixture. For the measurement of Savinase adsorption onto protein-soiled surfaces the so-called "AAPF" method proved to be applicable. This method is based on the detection of the proteolytic activity of Savinase on AAPF (N-succinyl-L-Alanyl-L-Alanyl-L-Prolyl-L-Phenylalanine-p-Nitroanilide). As autolysis of active protease becomes more rapid at higher enzyme concentrations this method is restricted to the concentration regime of 0.1 $\mu g\ ml^{-1}$ to 30 $\mu g\ ml^{-1}$.

In laundry washing detergent systems the enzymes have to adsorb onto soiled cloth in order to remove stains. These practical "solid" surfaces proved to be very complex as they are mostly porous, irreproducible and difficult to characterise. Therefore, the examination of the adsorption mechanism of both enzymes, the main part of this study, is performed using well characterised, non-porous and non-hydrolysable solid surfaces. The enzyme adsorption at these solid-liquid interfaces has been approached in a similar way as protein adsorption is generally studied. In Chapter 3 the adsorption of Savinase on glass and on several polystyrene latices is reported at various pH-values, ionic strengths and temperatures. The adsorption of Savinase at solid-water interfaces was found to be driven by electrostatic interactions between the surface and the enzyme, dehydration of hydrophobic interfaces and lateral interactions between the adsorbed Savinase molecules. It was also concluded that the enzyme adsorbed as a sphere and did not unfold upon adsorption.

As the actual adsorption depends strongly on the electrostatic interactions between the surface and the enzyme the contributions of electrostatic lateral repulsion and dehydration of hydrophobic parts of the surface to the free energy must be of the same order of magnitude. The range of the lateral repulsion extended far beyond the Debye length. The relative importance of the electrostatic interaction resulted in a decrease of adsorption on a negatively charged surface with an increase in pH. Under attractive electrostatic conditions the adsorbed amount decreased with increasing ionic strength. The uptake of an extra Ca^{2+} ion in the weak calcium binding site of Savinase increased the adsorption at a negatively charged interface, probably because of the increased positive charge on the enzyme. The weak binding site proved to be selective for calcium ions as magnesium ions were not sequestered.

The adsorption of Savinase is dynamic, i.e. protein molecules replaced already adsorbed ones, although it was not reversible against dilution. On a hydrophobic surface was it proved to be reversible towards changes in pH and ionic strength. The adsorption of inhibited Savinase was temperature independent in contrast to that of active Savinase at 30°C which was strongly determined by enzymatic autolysis.

In Chapter 4 the influence of the properties of Savinase on its adsorption on PS-latices and glass and on the interaction with chromatographic column materials is examined. Therefore, a set of six closely related protein-engineered variants of Savinase were studied. The variants differed from the naturally occurring Savinase in their electrostatic properties such as the isoelectric point and the number of charged amino-acids. On negatively charged PS-latex differences in electrostatic interaction dominated the differences between the adsorption of the various Savinase variants. The electrostatic properties of the variants could not be described completely on the basis of either the net charge of the protein or the mean surface potential. Altering the primary structure of the protein brought about changes that could not be described properly without specifically considering the distribution of the charged residues over the protein surface. Hydration of the enzyme was a less important factor for the adsorption on hydrophobic PS-latex especially in the case of electrostatic attraction.

The adsorption on PS-latex was compared with the retention on chromatographic columns and this proved to be useful in the investigation of the relative importance of electrostatic and hydrophobic interactions for protein adsorption. The retention of the Savinase variants on the hydrophobic interaction column (Alkyl-Superose®) showed that the retention time was inversely related to the polar - non-polar area ratio of the protein. The retention of the variants on a cation exchange column could not be described satisfactorily with the mean surface potential of the protein. A first examination of the relation between charge distribution over the protein and retention on a negatively charged column showed that strongly localised alterations of the potential could not be expected to lead to differences in retention.

In Chapter 5 the adsorption of Lipolase on glass and various polystyrene latices was examined. The approach was similar to that for Savinase, reported in Chapter 3. The adsorption mechanism of Lipolase also proved to be comparable to that of Savinase. Electrostatic interaction and dehydration of hydrophobic parts of the surfaces are the main driving forces. Under attractive electrostatic interaction between the surface and the enzyme the plateau value of the isotherm corresponds to a monolayer coverage (2.3 mg m^{-2}). Under experimental conditions in which dehydration of the hydrophobic surface is almost compensated by electrostatic repulsion the lateral repulsion between the adsorbed enzymes becomes important and determines the surface coverage of Lipolase. Just as in the case of Savinase it was concluded that Lipolase did not unfold significantly upon adsorption.

In the final chapter the examined experimental techniques and the insight into the driving forces for Savinase adsorption obtained, are applied to the adsorption of Savinase at

practical solid-liquid interfaces such as polyester, cotton and cotton artificially soiled with the protein BSA (Bovine Serum Albumin). These practical solid surfaces proved to be much more complex than the non-porous, non-hydrolysable, surfaces that were used in the previous chapters. A preliminary characterisation of these interfaces, based upon the adsorption of well known proteins, a determination of the time dependence of Savinase adsorption and the measurement of the isotherms under various conditions have been carried out. The specific surface area of polyester was determined to be 0.27 $m^2 g^{-1}$ by adsorption of the protein Lysozyme. Lysozyme adsorption on polyester reached equilibrium within 1 hour. Under electrostatically attractive conditions the maximum adsorption of Savinase on polyester corresponds to monolayer coverage. The affinity of Savinase for the polyester surface was determined by electrostatic interaction together with dehydration of the hydrophobic interface.

By Lysozyme adsorption measurement, the surface areas of clean and BSA-soiled cotton were determined to be 15 $m^2 g^{-1}$ and 9 $m^2 g^{-1}$, respectively. However, the actual values may differ by as much as 50% from the ones determined in this way as Lysozyme adsorption proved to be surface-specific. Adsorption equilibrium was reached after 8 hours contact time. During this period protein diffuses into the hollow cotton fibres. The adsorption of Savinase on clean cotton resembled that on glass and is mainly determined by electrostatic interaction between the enzyme and the surface. The uncertainty in the surface areas of clean and BSA-soiled cotton prevented the determination of possible preferential adsorption of Savinase onto protein soiled cotton.

The adsorption of active and PMSF inhibited Savinase on cotton artificially soiled with BSA differed very strongly even after short contact times. Although further experimental evidence is necessary we conclude that the lower adsorption on BSA-soiled cotton of active Savinase compared to that of PMSF inhibited Savinase was (partially) caused by the decreased affinity of the enzyme for the altered interface. Alteration of the solution by an increased concentration of hydrolysed BSA fragments did not significantly reduce the adsorption.

The adsorption mechanism of Savinase and Lipolase indicated above can be compared with that reported for other proteins. In the literature protein adsorption in general is described as governed by a delicate balance between four categories of interactions namely protein-surface interactions (electrostatic and Van der Waals interactions), the dehydration of the protein and solid surfaces, the structural alteration of the protein and the lateral interaction between the adsorbed proteins. As a first step, proteins can be divided into two categories, "soft" and "hard", according to the resilience against structural alterations (as discussed in Chapter 1). The results reported here show that

Savinase and Lipolase are even "harder" (more rigid) than the so-called "hard" proteins examined in the literature. The adsorption of "hard" proteins, such as RNase and Myoglobin, onto like charged hydrophobic surfaces is caused by their flattening upon adsorption. This flattening is the cause of an increase in the dehydrated surface area on the interface. This interaction is strong enough to compensate for the electrostatic and lateral repulsion. The rigidity of both enzymes examined here is thought to obstruct this flattening therewith limiting the dehydrated surface area. The absence of structural alterations and the relatively small contribution of dehydration make Savinase and Lipolase adsorption very sensitive to the other two important interactions: the electrostatic protein-surface interactions and the lateral interaction between the adsorbed proteins.

SAMENVATTING

ENZYM-ADSORPTIE VANUIT EEN OPLOSSING AAN EEN VASTE STOF

Enzymen zijn in de natuur van groot belang. Deze eiwitmoleculen versnellen vrijwel alle reacties in levende organismen. Twee enzymen worden in dit onderzoek nader bestudeerd, te weten Savinase™ en Lipolase™. Savinase is een extracellulaire serine protease van de bacterie *Bacillus lentus* en Lipolase een extracellulaire lipase van de schimmel *Humicola lanuginosa* S-38. Proteases katalyseren onder meer de afbraak van eiwitmoleculen; lipases bevorderen de ontleding van vetten. Zo spelen deze enzymen een rol bij de spijsvertering.

Proteases en lipases zijn ook bij het wassen van textiel van nut voor de verwijdering van vlekken. Om een optimaal gebruik van dit natuurlijke wasmiddel te kunnen maken, is inzicht vereist in de wijze waarop deze reiniger zijn werk doet. De enzymwerking kan worden gedacht uit drie fasen te bestaan. De eerste stap in het proces is de adsorptie van het enzym uit de oplossing naar de vuile was. Wanneer het enzym zich op het oppervlak heeft gehecht, 'knipt' het de eiwit- of vetvlek aan stukjes. Tenslotte is er de verwijdering van de ontstane brokstukken van het oppervlak naar de oplossing. Het onderzoek beschreven in dit proefschrift is beperkt tot de eerste fase, de adsorptie vanuit een oplossing aan een vaste stof.

Het alledaagse wasproces is buitengewoon gecompliceerd. Kleding bestaat onder meer uit katoen en polyester, waarvan de oppervlakken poreus zijn en moeilijk te karakteriseren. De werking van de enzymen wordt bovendien beïnvloed door de overige componenten van het wasmiddel. Om inzicht te verwerven in het mechanisme waarmee de enzymen vuil verwijderen, is gekozen voor een vereenvoudigd, maar toch representatief, systeem. In eerste instantie is gebruik gemaakt van schone, niet-poreuze oppervlakken, waarvan de kenmerken vanuit de literatuur welbekend zijn. Aan de enzym-oplossing zijn geen andere wasmiddelcomponenten toegevoegd.

Deze studie is erop gericht het mechanisme te begrijpen dat de enzym-adsorptie veroorzaakt. Welke krachten trekken het eiwit naar het oppervlak toe? Welke omstandigheden bevorderen of remmen dit proces? Het onderzoek dat is verricht om op deze vragen een antwoord te vinden, is in zes hoofdstukken beschreven. Na de inleiding in hoofdstuk 1, wordt in hoofdstuk 2 overwogen welke technieken voor het bepalen van eiwit-adsorptie beschikbaar zijn. In hoofdstuk 3 wordt door variatie van de experimentele omstandigheden het adsorptie-mechanisme van Savinase onderzocht. De resultaten uit

hoofdstuk 3 worden in hoofdstuk 4 getoetst door variatie van de protease zelf. Hoofdstuk 5 is gewijd aan de adsorptie van Lipolase. In hoofdstuk 6 tenslotte wordt getracht een verbinding te leggen met de complexe praktijk. De adsorptie van Savinase wordt bestudeerd niet aan model-oppervlakken doch aan lapjes polyester en katoen.

Meettechnieken

Om het mechanisme van enzym-adsorptie te doorgronden, is het nodig de geadsorbeerde hoeveelheid van het enzym te meten. De enzym-adsorptie wordt bepaald door de concentratie in de oplossing vast te stellen vóór en na het contact met het oppervlak. Daarvoor zijn in principe verschillende methoden beschikbaar.

Onder praktijkomstandigheden moet het Savinase adsorberen aan een met eiwit bevuild oppervlak. Daarom verdient een methode de voorkeur die het enzym van de verwijderde eiwit-brokstukken onderscheidt. Om die reden is overwogen gebruik te maken van de radioactieve tracermethode, waarbij aan een gedeelte van het enzym een radioactief merk wordt gehecht. Het proces van merken bleek evenwel het adsorptievermogen van het enzym aan te tasten. Een andere bekende methode voor de bepaling van de eiwit-concentratie is gebaseerd op meting van de extinctie bij een golflengte van 280 nm. Deze meetwijze bestrijkt evenwel niet het lage concentratiegebied van de isotherm, de curve die de afhankelijkheid aangeeft van de adsorptie ten opzichte van de concentratie van het in oplossing achterblijvende eiwit.

De eiwitkleuringsmethode van Lowry is bruikbaar zolang er slechts één eiwit in de oplossing aanwezig is. Ter aanvulling kan echter de AAPF-methode dienen, die de activiteit van het enzym toont ten aanzien van het modelsubstraat AAPF. Bij de inwerking van het enzym op dit AAPF komt een kleurstof vrij waarvan de hoeveelheid via spectrofotometrie kan worden vastgesteld. Op deze wijze wordt een ijklijn verkregen ter bepaling van de Savinase-concentratie. Van deze beide methoden is in dit onderzoek gebruik gemaakt.

De adsorptiebepaling van de protease wordt bemoeilijkt door het feit, dat dit enzym alle eiwitten ontleedt, dus ook zichzelf aantast, hetgeen autolyse wordt genoemd. Om deze autolyse te ondervangen wordt gebruik gemaakt van 'geremd' Savinase, waarbij de enzymatische activiteit is verminderd door binding aan het enzym van PMSF. Deze remming blijkt het adsorptie-vermogen niet te beïnvloeden.

Oppervlakken en meetomstandigheden gevarieerd

In de literatuur wordt eiwitadsorptie toegeschreven aan de combinatie van vier krachten: de electrostatische wisselwerking, de afstoting van water door de hydrofobe delen van eiwit en oppervlak, de laterale repulsie tussen de gelijkgeladen geadsorbeerde eiwitmoleculen, en de toename van de wanorde, de entropie, door ontvouwing van het eiwit bij adsorptie. Om vast te stellen in welke mate de verschillende krachten het proces beïnvloeden, zijn de isothermen bepaald van de adsorptie aan verschillende oppervlakken en onder gevarieerde meetomstandigheden. Positief en negatief geladen polystyreen latex zijn gekozen als hydrofobe oppervlakken en glas als negatief geladen hydrofiel oppervlak. De condities zijn gewijzigd door wisseling van de pH, de aard en concentratie van de ionen en de temperatuur.

Van de vier genoemde factoren blijkt de ontvouwing hoegenaamd geen rol te spelen. De toename van de entropie van het eiwit is daarmee als drijvende kracht te verwaarlozen. Doordat het eiwit zich niet ontvouwt, blijft ook het raakvlak met het oppervlak klein, hetgeen de hydrofobe wisselwerking van minder belang maakt. Het feit dat de genoemde factoren in het proces een relatief kleine rol spelen, maakt dat de adsorptie van Savinase en Lipolase vooral wordt bepaald door de electrostatische wisselwerking en de laterale afstoting.

Het raakvlak lijkt zo klein te zijn, dat we moeten concluderen dat de enzymen niet alleen hun conformatie behouden, maar ook bij contact met het oppervlak niet worden afgeplat. Zelfs het in de literatuur als 'hard' gecategoriseerde RNase, drukt over een groter gedeelte van het oppervlak het water weg. Dit kan worden afgeleid uit het feit, dat bij vergelijkbare electrostatische afstoting RNase wèl aan hydrofobe oppervlakken adsorbeert en Savinase en Lipolase dat niet doen. Bij de hier onderzochte enzymen is de hydrofobe interactie dus niet groot genoeg om de electrostatische afstoting te overwinnen.

Daar de electrostatische krachten van overwegend belang blijken, dringt zich de vraag op welke omstandigheden de adsorptie bevorderen. In het voor het wasproces relevante pH-gebied, tussen 7 en 11, is Lipolase negatief geladen. Savinase daarentegen is bij een pH onder 10 positief en daarboven negatief. Tussen het positieve Savinase en het negatief geladen oppervlak wordt de aantrekking groter bij dalende pH resp. afnemende ionenconcentratie. Dientengevolge neemt de adsorptie toe. Ook bij het negatieve Lipolase wordt de adsorptie bevorderd door een dalende pH en een stijgende concentratie ionen, in dit geval ten gevolge van een afnemende afstoting.

Zowel voor Savinase als voor Lipolase geldt dat de adsorptie aan een negatief geladen oppervlak toeneemt bij toevoeging van calcium-ionen aan de oplossing. Bij Savinase kon worden aangetoond, dat dit effect wordt veroorzaakt door de specifieke binding van calcium aan het enzym, hetgeen diens positieve lading vergroot.

De hierboven vastgestelde krachten die de adsorptie van Savinase en Lipolase bewerken, zijn, naar het zich laat aanzien, niet zeer sterk. De verschillende interacties houden elkaar ongeveer in balans. Een kleine variatie in de experimentele omstandigheden kan daardoor grote veranderingen teweegbrengen. Dit blijkt onder meer uit het feit, dat ook na adsorptie een verandering van de pH of van de ionenconcentratie tot een eindtoestand leidt, die in overeenstemming is met de uiteindelijke omstandigheden. Zelfs bij een hydrofoob oppervlak vermindert of vermeerdert de geadsorbeerde hoeveelheid naargelang de omstandigheden. Het geadsorbeerde eiwit blijkt ook door een ander eiwit te kunnen worden vervangen. Het feit tenslotte dat het adsorptieproces onafhankelijk is van de temperatuur, wijst er eveneens op dat de optredende krachten gering zijn.

Varianten van het enzym Savinase

Eiwitten worden door de natuur aangepast aan de verschillende omstandigheden waaronder ze actief moeten zijn. Een eiwit bestaat uit een keten van aminozuren. Van deze bouwstenen bestaan slechts twintig typen. Doordat echter een eiwit uit een groot aantal aminozuren is opgebouwd, is de variatie in eiwitten groot. Een aanpassing nu kan worden getypeerd als een uitwisseling van een of meer aminozuren. Deze aanpassingen kunnen tegenwoordig ook onder laboratoriumomstandigheden worden bewerkstelligd.

Aminozuren zijn positief of negatief geladen dan wel ongeladen. De netto-lading van een eiwit is gelijk aan het verschil van het aantal positieve en negatieve bouwstenen. Daar de electrostatica een van de drijvende krachten van adsorptie is gebleken, hebben wij gebruik gemaakt van twee groepen van in totaal zes Savinase-varianten, die wat betreft hun electrostatische eigenschappen verschillen. In de ene groep varieert de netto-lading, terwijl het aantal geladen aminozuren gelijk blijft. In de andere groep verschilt, bij constante netto-lading, het aantal geladen bouwstenen.

De nettolading blijkt, zoals op grond van de resultaten in hoofdstuk 3 mocht worden verwacht, een factor van belang te zijn. Een toenemende netto-lading van het enzym correspondeert met een toenemende adsorptie aan een tegengesteld geladen oppervlak. De varianten met verschillende aantallen geladen aminozuren laten zien, dat de adsorptie aan een ongeladen oppervlak beïnvloed wordt door de oplosbaarheid. Met het aantal geladen

bouwstenen neemt de affiniteit van het enzym voor het oplosmiddel toe, hetgeen de adsorptie vermindert.

Niet alle gevolgen van variatie in ladingspunten echter blijken vanuit de oplosbaarheid te verklaren. Bij een geladen oppervlak brengen de varianten significante verschillen aan het licht, die niet corresponderen met de veranderingen in adsorptie bij ongeladen oppervlakken. Mogelijkerwijs is de ladingsverdeling hiervoor verantwoordelijk. Om deze hypothese te toetsen is de localisering van de geladen aminozuren per eiwitmolecuul in kaart gebracht. De interpretatie van de ladingsdistributie in relatie tot adsorptie is echter nog in het stadium van discussie. Een afdoende verklaring voor de geconstateerde verschillen kan nog niet worden geboden.

Adsorptie aan polyester en katoen

Tot nu toe is de adsorptie bestudeerd aan goed gekarakteriseerde oppervlakken. In de praktijk van de textielwas echter hebben we met poreuze, in de tijd veranderende stoffen te doen, zoals katoen en polyester. Om een eerste verkenning mogelijk te maken, moet de vraag worden beantwoord, hoe de grootte van het poreuze oppervlak van deze stoffen kan worden bepaald.

De verschillende methoden die voor de oppervlaktebepaling worden gehanteerd, schieten voor het hier beoogde doel tekort. Bij gasadsorptiemetingen wordt het oppervlak van de textiel ongewild verkleind, doordat de poreuze structuur tengevolge van de lage druk ineenstort. Oppervlaktemeting door adsorptie van kleine moleculen uit een oplossing leidt tot een voor de grote eiwitmoleculen irrelevant hoge waarde. Aangewezen zijn we derhalve op adsorptiemetingen met moleculen van vergelijkbare grootte als het Savinase. Hiertoe is gekozen voor Lysozyme, dat volgens de literatuur op verschillende oppervlakken gelijkelijk zou adsorberen.

Op deze wijze kon het oppervlak van polyester op 0,27 m^2 per gram worden vastgesteld, van schone katoen op 15 m^2 per gram en van katoen bevuild met het eiwit BSA op 9 m^2 per gram. Vanwege het feit dat Lysozyme toch tot op zekere hoogte afhankelijk bleek van de aard van het oppervlak, moet met een onzekerheid in de orde van 50% rekening worden gehouden.

De adsorptie van Savinase aan polyester bereikt binnen een uur zijn evenwichtswaarde. De affiniteit van Savinase voor dit oppervlak wordt in hoofdzaak bepaald door electrostatische wisselwerking aangevuld met hydrofobe wisselwerking tussen enzym en polyester. Ten gevolge van de grote porositeit van het katoen duurt het meer dan acht

uur, voordat evenwicht is bereikt. De adsorptie op schone katoen wordt gestuurd door electrostatische interactie.

De adsorptie aan met eiwit bevuilde katoen wordt sterk verminderd door de optredende enzymatische activiteit; het enzym is immers bedoeld om de eiwitlaag aan te tasten. Gebleken is, dat deze verlaging van de adsorptie niet kan worden geweten aan het feit dat de oplossing vervuild raakt met van het oppervlak afkomstige eiwitfragmenten. Door de afbraak van het eiwit op het katoen veranderen de eigenschappen van het oppervlak. De sterke vermindering van de adsorptie van Savinase op bevuild katoen kan aan deze veranderingen worden toegeschreven.

CURRICULUM VITAE

Susan Duinhoven, op 6 juli 1965 te Amsterdam geboren, behaalde het VWO-diploma in 1983 aan het Willem de Zwijger College te Bussum.

Van 1983 tot 1988 studeerde zij scheikunde aan de Vrije Universiteit en aan de Universiteit van Amsterdam. Binnen haar bijvakstudie, theoretische chemie, heeft zij ab-initio-berekeningen aan BeH_2, H_2O en F_2 uitgevoerd binnen de vakgroep van Prof.Dr. E.J. Baerends, Vrije Universiteit. Voor het hoofdvak fysische chemie heeft zij onder leiding van Prof.Dr. R.P.H. Rettschnick, verbonden aan de Universiteit van Amsterdam, moleculair spectroscopisch onderzoek gedaan naar de configuratie van het Van der Waals-complex tetrazine-N_2 in een supersone molecuulbundel. Aan de Universiteit van Amsterdam is zij op 18 mei 1988 cum laude afgestudeerd.

Van juni 1988 tot en met mei 1992 was zij in dienst van het Unilever Research Laboratorium te Vlaardingen. In deze periode heeft zij onderzoek verricht naar het adsorptie-mechanisme van enzymen vanuit een oplossing aan een vaste stof. Bij dit onderzoek, dat onderwerp is van dit proefschift, werd zij begeleid door Dr. W.G.M. Agterof (Unilever Research Laboratorium), Prof.Dr. J. Lyklema (promotor, LU Wageningen) en Dr. W. Norde (co-promotor, LU Wageningen).

Sinds 1 juni 1992 is zij werkzaam bij Lever Nederland B.V. te Capelle aan den IJssel.

Rotterdam, augustus 1992