DOWNSTREAM PROCESSING OF POLYSACCHARIDE DEGRADING ENZYMES BY AFFINITY CHROMATOGRAPHY

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Aan mijn vader en moeder

Aan Margot

STELLINGEN

1. Mellor et al. gaan bij de beschrijving van een bioreaktor voor de reductie van nitraat en nitriet voorbij aan de aanwezigheid van NO-reductase in de beschreven reaktieketen.

R.B. Mellor, J. Ronnenberg, W.H. Campbell en S. Diekmann, Nature 355, 717-719 (1992)
A.H. Stouthamer, Journal of Bioenergetics and Biomembranes 23, 163-185 (1991)

2. Op basis van de uitgevoerde enzymaktiviteitsmetingen voor ß-galactosidase tijdens de opwerking van het enzym uit een complex medium kunnen geen conclusies getrokken worden met betrekking tot het mechanisme achter de aktivering van het enzym.

A-K. Frej, J-G. Gustafsson en P. Hedman, Biotechnology and Bioengineering 28, 133-137 (1986)

3. De door Ettalibi en Baratti gegeven resultaten voor de immobilisatie van inulinases op poreus glas komen niet overeen met de door hen gegeven experimentele condities en de door hen gegeven hoeveelheden aan de drager geadsorbeerd eiwit.

M. Ettalibi en J.C. Baratti, Biocatalysis 5, 175-182 (1992)

4. De in het rapport "Aanbevelingen voor het monitoren van stoffen van de M-lijst uit de derde Nota Waterhuishouding" gegeven berekeningswijze van de hoeveelheid organische stof uit de hoeveelheid organisch koolstof is op grond van de door de auteurs aangehaalde literatuur niet overtuigend.

CUWVO, Aanbevelingen voor het monitoren van stoffen van de M-lijst uit de derde Nota Waterhuishouding, Den Haag (1990)

5. De door Gardiner et al. gegeven relatie tussen de temperatuur en de dampspanning voor twee barium-ß-diketonaat polyethercomplexen is niet in overeenstemming met hun eigen resultaten.

R. Gardiner, D.W. Brown, P.S. Kirlin en A.L. Rheingold, Chem. Mater. 3, 1053-1059 (1991)

6. De methodiek van Reddi om met behulp van het 3-methyl-2-benzothiazolon hydrazon reagens specifiek heparansulfaat in urine te bepalen is onjuist.

A.S. Reddi, Clin. Chim. Acta 189, 211-220 (1990)

7. Het experimentele gegeven dat intratracheale instillatie van elastase leidt tot emfysemateuze laesies in proefdieren impliceert niet dat elastine de sleutelcomponent is bij de pathogenese van pulmonair emfyseem.

E.C. Lucey, P.J. Stone en G.L. Snider, In: "The Lung; Scientific Foundations", (R.G. Crystal, J.B. West et al., Eds.), Raven Press Ltd., New York (1991) pp. 1789-1801

8. Een gedegen kennis van de bijbelse geschiedenis is noodzakelijk voor een goede beschouwing van christelijke middeleeuwse architectuur.

9. Een lange auteurslijst boven wetenschappelijke artikelen is vaak meer een aanwijzing voor de aanwezigheid van een multidisciplinair samenwerkingsverband dan voor een kwantitatief aanzienlijke inbreng van alle auteurs.

10. De EO geeft met zijn gospel country uur mogelijkerwijs nieuwe impulsen aan het onderzoek naar de tot op heden niet iconografisch te verklaren middeleeuwse afbeelding van Christus te paard.

11. De opdracht in proefschriften zou door veel promovendi graag aangevuld worden met "en voor mezelf".

Stellingen behorende bij het proefschrift "Downstream Processing of Polysaccharide Degrading Enzymes by Affinity Chromatography".

W.A.C. Somers
Wageningen, 11 december 1992

CONTENTS

CHAPTER 1

RECOVERY OF (POLY)SACCHARIDE DEGRADING ENZYMES

This chapter has been published as part of the review:
W. Somers, J. Visser, F.M. Rombouts and K. van 't Riet (1989) "Developments in downstream processing of (poly)saccharide converting enzymes", J. Biotechnol. 11, 199-222

1.1 INTRODUCTION

In the last decades enzyme separation and purification has become increasingly important because of the evolving application of enzymes in the brewing, food, textile, chemical, detergent and pharmaceutical industries. Together with these developments improved separation techniques were developed. Production and purification of enzymes are strongly dependent on the potential market, the processing costs, the quality required and the available technology. Two main product categories can be recognized:

1. Pure preparations, mainly in use in research and analytical applications
2. Crude or partially purified industrial enzymes (e.g. proteases and polysaccharidases), mainly in use in industrial applications.

Most of the enzymes in the first category are purified by chromatographic techniques after a crude isolation, whereas most of the enzymes in category two are isolated by precipitation and membrane separation. An important application area for enzymes is food processing. It can be expected that applications in the food area will require an increasing level of purification in the future due to trends in this industry and to stricter commodity law demands.

Recently novel purification techniques have been developed, based on liquid-liquid extractions and affinity interactions. Although they show considerable potential for the purification of several industrial enzymes they have not been widely implanted in existing processes yet. The introduction and application of these techniques in the field of (poly)saccharidases will be discussed in this paper.

1.2 (POLY)SACCHARIDE CONVERTING ENZYMES

Among the group of industrially prepared bioproducts, enzymes have a relatively modest position. As shown in Table 1.1 the market value for bulk enzymes was estimated to be US $ 265 million in 1981. The predominant groups in this field are those of the proteases and the (poly)saccharide converting enzymes, which find their main applications in the food and beverage industry (Table 1.2). The industrially important saccharide converting enzymes can be classified in various groups which will be discussed briefly in this section. These groups are: starch degrading enzymes, pectic enzymes, cellulases and monosaccharide converting enzymes (e.g. glucose oxidase, glucose isomerase, ß-

galactosidase and invertase).

Table 1.1 - World-wide production of enzymes in 1981 in relation to other bioproducts (adapted from ref. [1]).

Commodity	Value (US $, millions)
Antibiotics	6300
Ethanol	2100
Organic acids	500
Amino acids	500
Enzymes, industrial (bulk)	265
Enzymes, analytical and therapeutic	35

Starch degrading enzymes

The granules of common starches contain in general 15-30% amylose and 70-85% amylopectin. Amylose is a linear chain of 500 or more glucose units joined by α-1,4-glycosidic linkages. The molecular size differs in different species. Amylopectin is a glucose polymer consisting of a branched configuration in which linear chains (comparable with amylose) are interconnected by α-1,6 linkages at the branch points [2].

Starch degrading enzymes are wide-spread and are produced by microbes and by plant and animal tissues. In the group of amylases several important enzymes can be distinguished:

1. Exo-acting enzymes

a. Amyloglucosidases (E.C. 3.2.1.3) or glucoamylases hydrolyse α-1,4 and α-1,6 linkages, the endproduct of the conversion of starch being glucose.

b. ß-amylases (E.C. 3.2.1.2) hydrolyse the α-1,4 bonds in the starch molecule. They can not bypass α-1,6 linkages in amylopectin. Therefore the degradation of amylopectin by this enzyme is not complete. The chains are degraded up to the branches yielding 50-60% maltose [3]. The remaining fraction is called a ß-limit dextrin.

2. Endo-acting enzymes

α-amylases (E.C. 3.2.1.1) hydrolyse α-1,4 bonds and bypass α-1,6 linkages in the substrate. Hydrolysis of amylose goes through a series of oligosaccharides and finally yields maltose and maltotriose. Maltotriose, which is a poor substrate, can ultimately be converted to maltose and glucose. Breakdown

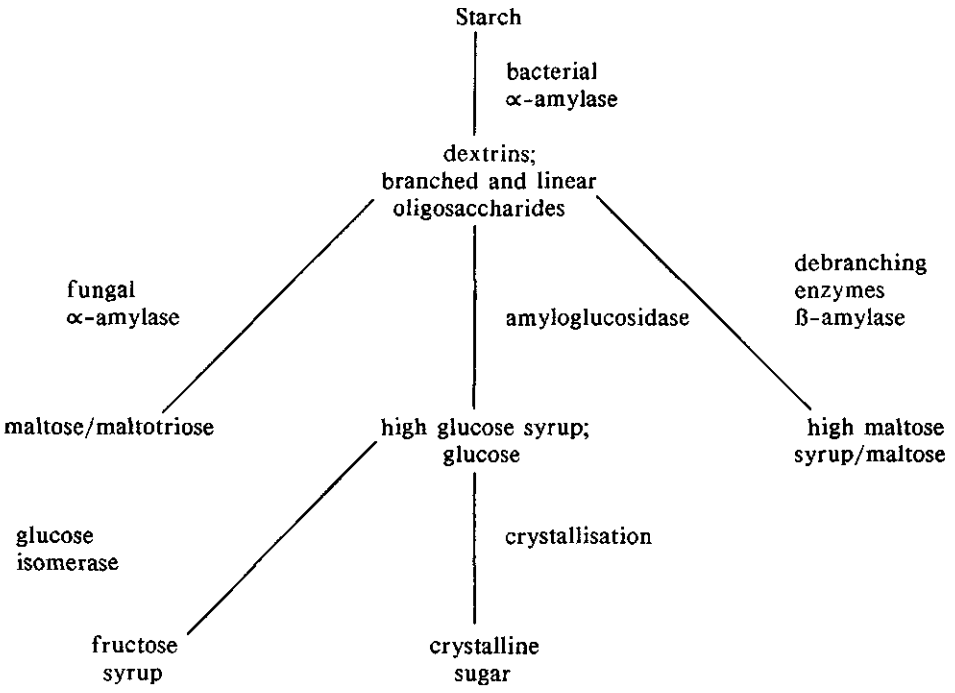
Table 1.2 - Main industrial enzymes and applications (adapted from ref. [1,4]).

Market	Enzyme	Application(s)
Starch	α-amylase	conversion of starch into dextrins
	amyloglucosidase glucose isomerase	production of fructose-syrups from dextrins
	ß-amylase	conversion of starch into maltose
Sucrose	invertase	conversion of sucrose into glucose and fructose
Dairy	ß-galactosidase	hydrolysis of lactose, improvement of quality and digestion of products
Beverages	pectinases	clarification, product and yield improvement of juices
	glucose oxidase + catalase	removal of oxygen from beverages, removal of glucose from egg products
Baking	α-amylase proteases hemicellulases amyloglucosidase	bread dough modification, flour supplementation
Laundry aids	alkaline protease α-amylase	presoak and washing, spot removal
Leather	protease	bating
Textile	α-amylase	starch desizing (e.g. cotton)

of amylopectin by α-amylase yields glucose and maltose combined with α-limit dextrins.

3. Direct debranching enzymes

This group of enzymes hydrolyses α-1,6 linkages in amylopectin and/or glycogen. There are two debranching enzymes which react with unmodified amylopectin: isoamylase (E.C. 3.2.1.68) and pullulanase (E.C. 3.2.1.41). The main difference between these enzymes is that pullulanase is able to hydrolyse the α-glucan pullulan whereas isoamylase can not convert this substrate.



Scheme 1.1 - Conversion of starch by starch degrading enzymes.

Applications

In the starch processing industry these enzymes are widely used for conversion of the substrate into sugars, syrups and dextrins. The products find a wide application in the food and beverage industry and can be used for fermentation processes. Scheme 1.1 shows the conversion of starch into its various products.

Pectic enzymes

Pectic substances, the main substrates for this group of enzymes, are found in a variety of plant materials. The pectins consist predominantly of chains of partially methylesterified α-1,4-D-galacturonic acid residues with a molecular weight of 30,000 up to 300,000 Da. The demethylated form is known as pectic acid or polygalacturonic acid [5].

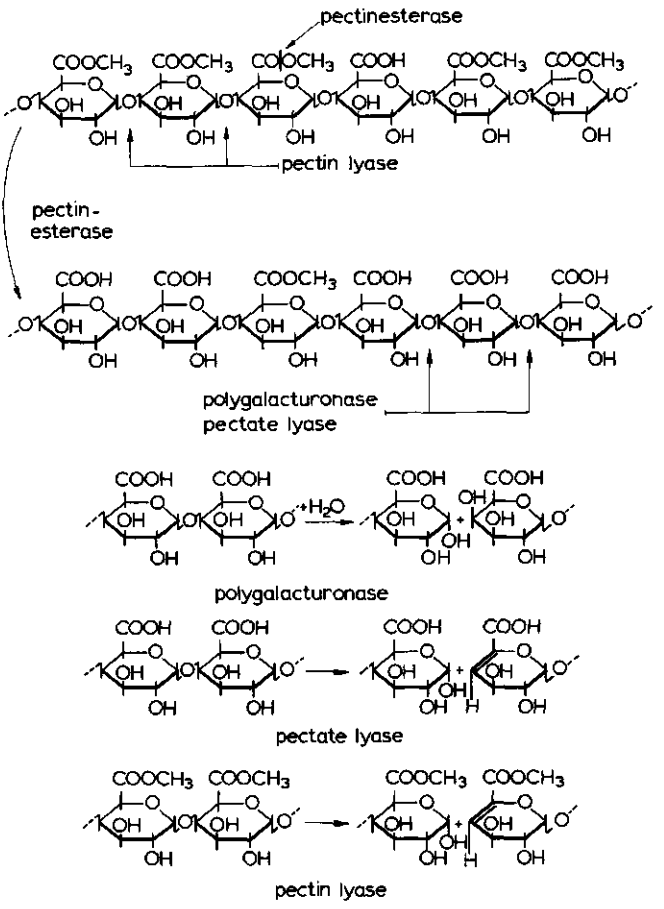


Fig. 1.1 - Pectic enzymes and their mode of action.

Pectic enzymes are generally classified in three groups:

1. pectinesterases (E.C. 3.1.1.11) which deesterify pectin;

2. chain-splitting or depolymerizing enzymes which split the α-1,4 linkages in the preferred pectic substrates by hydrolysis (hydrolases or polygalac-

turonases) and

3. depolymerizing enzymes with C_4-C_5 ß-elimination activity (lyases) (see Fig. 1.1).

In the group of hydrolases endo-polygalacturonase (E.C. 3.2.1.15) and exo-polygalacturonase (E.C. 3.2.1.67) can be distinguished by their mode of action. Endo-polygalacturonases hydrolyse pectate, their preferred substrate, randomly, the reaction products being oligogalacturonates (ultimately mainly mono- and trigalacturonates). The exo-polygalacturonases liberate galacturonate residues by a terminal attack on the polymer.

The group of lyases is characterized by the ß-eliminative attack on their substrates resulting in a double bond between C-4 and C-5 in the monomer of the released nonreducing end (see Fig. 1.1). Endo-pectate lyases (E.C. 4.2.2.2), split pectates and low methoxyl-pectates, whereas endo-pectin lyases (E.C. 4.2.2.10) can specifically depolymerize high methoxyl pectins. In the reaction process unsaturated oligogalacturonates are accumulating, the unsaturated dimer and trimer being the predominant ones [5]. Exo-pectate lyases (E.C. 4.2.2.9) liberate unsaturated dimers from the reducing end of the pectate.

Applications

Pectinases are used in various important processes especially in the beverage industry, the main application being in fruit-juice clarification. Addition of pectinases to pressed juices, which are normally viscous and turbid, results in a rapid drop of viscosity and clarification of the juice. Furthermore they are used in fruit-juice extraction, maceration and liquefaction of fruits and in the citrus technology as a valuable aid in the isolation of byproducts from fruitpulp (Rombouts and Pilnik, 1980).

Cellulases

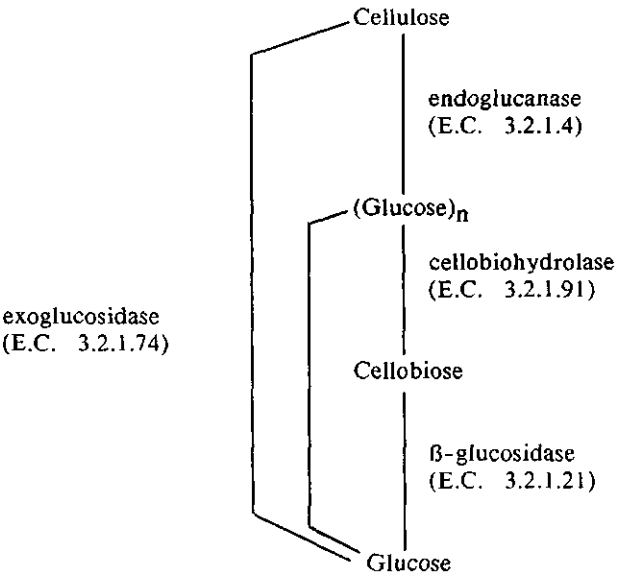
Cellulose is a glucose polymer, composed of anhydroglucose residues coupled by ß-1,4-glucosidic bonds. It is the most important constituent of cell walls and therefore wide-spread. The degree of polymerization may vary between 15 and 14,000. The cellulose molecules are connected to each other by hydrogen bonds giving rise to large crystalline regions [6]. The resulting fibril structure gives the material special chemical and physical properties; it is very rigid and almost inaccessible for enzymatic breakdown.

Cellulase (i.e. cellulolytic enzymes) activity is defined as the hydrolysis of cellulose. In this process several enzymes participate and a breakdown path for cellulose is given in Scheme 1.2. Endo-glucanases randomly hydrolyse ß-1,4-glucosidic linkages. Cellobiohydrolases act on cellulose splitting off cellobiose

units from the non-reducing end of the chain whereas ß-glucosidase hydrolyses cellobiose and cello-oligosaccharides to glucose. A minor activity of glucan-glucohydrolase is reported that removes glucose residues from the non-reducing end of the chain.

Applications

Cellulases have not been employed very widely in the food-processing industry although the possibility of production of glucose from cellulolytic materials would be very attractive. Applications however are not profitable at this moment due to the relatively high cost and the poor efficiency of the enzyme preparations. Improvements can be expected by the introduction of better enzyme systems or by improving the substrate accessibility for the enzymes for instance by compression, bead milling, acid hydrolysis, steaming or extrusion.



Scheme 1.2 - Cellulolytic enzymes and their conversions (adapted from ref.[7]).

Monosaccharide metabolizing enzymes

In Table 1.3 the reactions, catalyzed by this group of enzymes, are summarized.

Applications

The main application of glucose oxidase is in clinical diagnosis as an indicator in carbohydrate (i.e. glucose-) metabolism. Furthermore it is used in the beverage industry to remove oxygen from juices and to prevent oxidation [1]. Glucose isomerase is widely used in the conversion of glucose into fructose thus yielding syrups with yields of 42% in terms of fructose. ß-Galactosidase is used for the hydrolysis of lactose in skim milk to provide products that are (more) easily digestible. Furthermore it is used in analytical applications (e.g. isolation of fusion proteins from cell extracts [8]). Invertase is used for the conversion of sucrose in glucose and fructose, ingredients for the production of sweet foods.

Table 1.3 - Monosaccharide degrading enzymes.

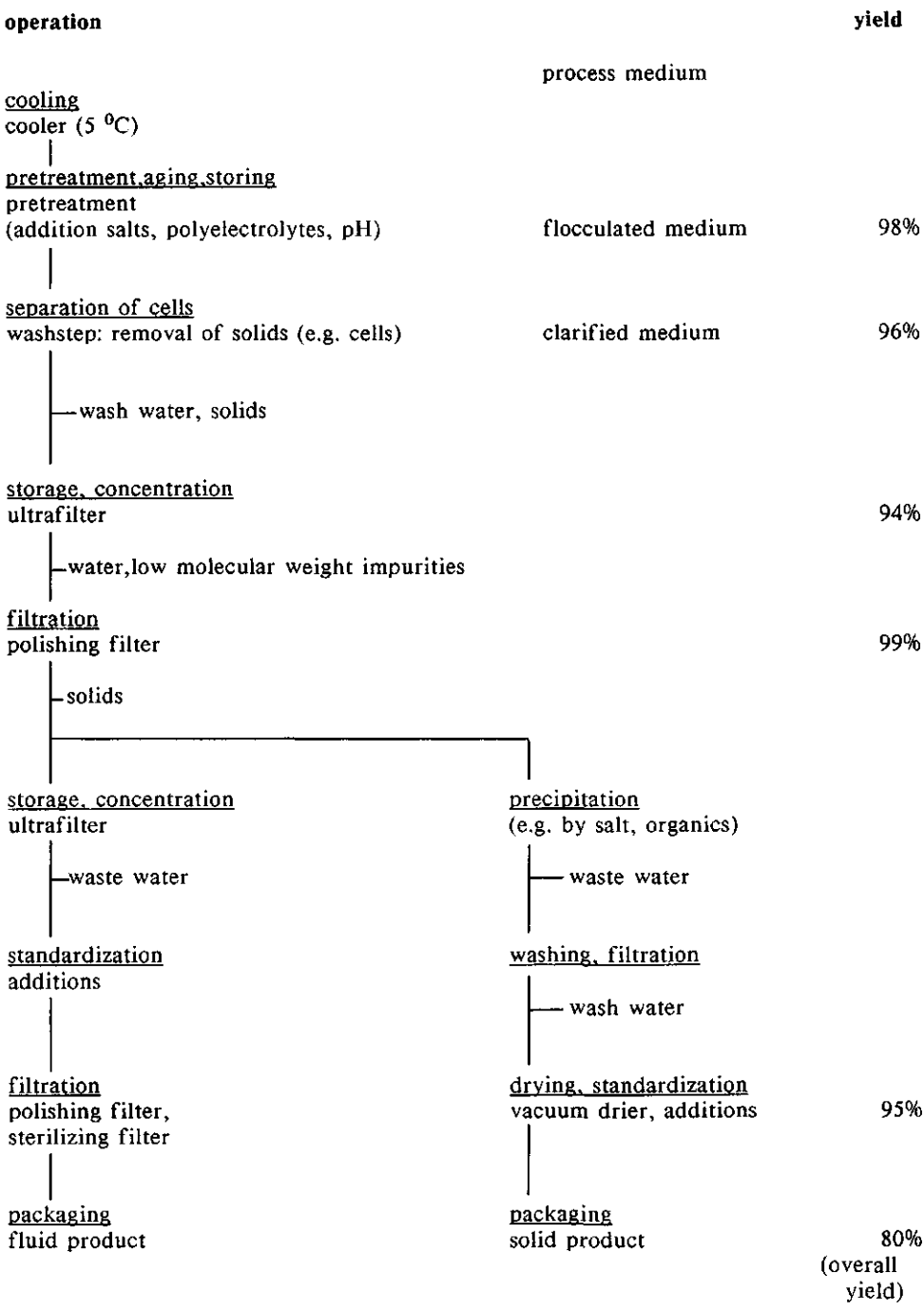
Enzyme	E.C. Number	Catalyzed reaction		
Glucose oxidase	(E.C. 1.1.3.4)	glucose	⇌	glucono-δ-lactone
Glucose isomerase	(E.C. 5.3.1.5)	glucose	⇌	fructose
β-Galactosidase	(E.C. 3.2.1.23)	lactose	⇌	glucose + galactose
Invertase	(E.C. 3.2.1.26)	sucrose	⇌	glucose + fructose

1.3 DOWNSTREAM PROCESSING OF SACCHARIDE CONVERTING ENZYMES

Purification of crude bulk enzymes

After synthesis the enzymes have to be recovered from the process medium. General reviews for downstream processing of proteins are given by refs. [9-14].

Although specific details about industrial downstream processes of enzymes are not available in literature, the isolation of bulk enzymes generally follows the routes for large scale separations of intra- and extracellular proteins respectively as proposed in ref. [10,13,14].



Scheme 1.3(a) - Process path for recovery of extracellular enzymes (e.g. protease) (adapted from ref. [10]).

operation	yield
process medium	
cell separation centrifuge	99%
├— waste medium	
cell disruption homogenizer/bead mill	96%
separation of solids cross flow filtration, centrifuge	93%
├— waste water	
concentration ultrafilter	98%
├— waste water	
precipitation addition of salts, nucleic acid precipitation	
filtration	
├— solids	98%
product precipitation	
filtration pressure filter	
drying, standardization vacuum evaporator, additions	95%
packaging product	80% (overall yield)

Scheme 1.3(b) - Process path for recovery of intracellular enzymes (e.g. amidase, ß-galactosidase) (adapted from ref. [10]).

Scheme 1.3 shows the flow sheets for the purification of an extracellular enzyme (protease) (Scheme 1.3(a)) and two intracellular enzymes (amidase and ß-galactosidase) (Scheme 1.3(b)) together with the stages and unit operations involved in the current industrial downstream processes of these proteins [10]. In the separation process usually only a limited number of unit operations is applied. These will be discussed below.

Cell disruption

In this step intracellularly produced proteins are released from the cells. Cell disruption is commonly achieved by (i) high shear, developed in mills and/or homogenizers; (ii) sonification, resulting in pressure waves; (iii) enzymatic degradation of cell walls (e.g. lysozyme), often restricted by the high costs and high amounts of lytic enzyme needed; (iv) chemical methods, (i.e. treatment with alkali, organic solvents, detergents or osmotic shock); (v) repeated freezing and thawing.

Solid-liquid separations

Solid-liquid separation is an important requirement for protein recovery. There are three main stages in the process where this is applied (see Scheme 1.3).

(i) separation of insolubles (cells and cell debris) from protein solutions or fermentation media;

(ii) separation of precipitated impurities from the liquid phase (e.g. nucleic acids);

(iii) separation of the precipitated product(s) from solutions.

Most commonly these separations are achieved by centrifugation, settling and flotation or filtration (rotary filtration, filter press filtration or membrane filtration [13,14].

Ultrafiltration and reverse osmosis

Ultrafiltration is used to remove water, inorganic and small organic molecules from protein solutions. This technique can be used in protein concentration and primary purification. The important advantages are minimum usage of chemicals, low process temperatures, no phase transition and easy scale-up. The most important field of application for ultrafiltration separations is the concentration of extracellular proteins. Reverse osmosis is usually used to concentrate protein solutions without purification [14].

The initial separation process of technical enzymes usually contains a

cascade of the steps discussed above and yields relatively pure preparations, the overall yield being circa 80%. As for the polysaccharide degrading enzymes this is often the end stage of purification. The commercial enzyme preparations generally contain several enzymes and therefore various enzyme activities accompany the main activity [15-17].

In the further purification and formulation of the product a number of operations can be involved like adsorption chromatography, crystallisation and precipitation by salting out or by solvent addition to the medium. These steps result in a more defined product.

Developments and their application in current industrial processes

Introduction of more specific or economically more attractive techniques in the industrial separation may yield more feasible processes. Table 1.4 summarizes the unit operations and their proposed alternatives involved in the isolation of intra- and/or extracellular proteins.

Direct recovery of the product from the process medium

Direct product recovery from the whole broth may be effected in different ways:

(i) Extraction of the desired protein from the whole broth. Here aqueous two-phase systems or reversed micellar extraction may be advantageous [18,19], and

(ii) Direct adsorption of products on adsorbents. The adsorbents must be large and dense enough to be separated from the medium solids. This can for instance be achieved in a fluidized bed reactor [20-22]. Special attention should be paid to the elution, reusability and to the mechanical stability of the adsorbent. For bulk enzymes an extra demand is that the adsorbent should be cheap, in relation to the price of the product.

(iii) Removal of the product from the medium through semi-permeable membranes. This offers the possibility to remove the product directly from the medium but also to remove the product during the process [23].

Recovery of proteins from clarified aqueous fractions

The operations described in the section above can also be applied to clarified process media. Other techniques that require clarified media (e.g. packed bed chromatography) can be implanted in a process at this stage. The most important examples in the recovery of (poly)saccharide converting enzymes include aqueous two-phase extractions, reversed micellar extraction, affinity separations, industrial isoelectrofocusing, membrane separation

Table 1.4 - Downstream processing of intra- and extracellular proteins, unit operations and their proposed alternatives - application to (poly)saccharide converting enzymes.

Operations	Enzyme type[a]	alternative/ improvement	Applications	
			Enzyme	ref
1.Recovery during synthesis	e	2-phase extraction; adsorption chromatography; reversed micellar extraction.	cellulase	24
2.Cell disruption	i,cb			
3.Flocculation or pretreatment	i,e,cb	ultrafiltration in presence of solids.	α-amylase ß-galactosidase	25 23
4.Solids separation; rotary or press filter; centrifugation	i,e,cb	direct enzyme recovery from broth;		
		2-phase extraction;	α-amylase pullulanase ß-galactosidase	26-28
		reversed micellar extraction;	α-amylase	19
		affinity adsorption;	endo-polygalacturonase[b]	
		direct precipitation.	-	
5.Concentration filtrate (ultrafiltration, vacuum evaporation)	i,e			
6.Precipitation by salt/solvent; filtration; drying	i,e,cb	recovery of protein from concentrate as "fixed" product on support.	ß-galactosidase	29
		large scale separations from clarified solutions (improved purity/better defined product) e.g.		
		affinity techniques;	several[b]	
		2-phase extraction;	several	26-28
		affinity ultrafiltration;	ß-galactosidase	23
		industrial autofocusing.	α-amylase	30

[a]: Enzyme types are defined as: i: intracellular enzymes; e:extracellular enzymes; cb: cell-bound enzymes [b]: see Section "Affinity chromatography".

processes and several chromatographic methods (for references see Table 1.4). In the following section the application of these techniques in the recovery of (poly)saccharide degrading enzymes is discussed with respect to their specificity and the stage where they can be implanted in the downstream process.

1.4 AFFINITY INTERACTIONS IN DOWNSTREAM PROCESSING OF (POLY)-SACCHARIDE CONVERTING ENZYMES

Affinity chromatography

The characteristics required for large scale affinity separations have been extensively reviewed [31-33]. The ideal matrix should have the following properties: (i) insoluble and chemically stable; (ii) hydrophylic and inert; (iii) porous and permeable; (iv) rigid, resistant to compression; (v) easily derivatizable, possessing an adequate number of functional groups and (vi) inexpensive and reusable.

Affinity separations of (poly)saccharide degrading enzymes

Affinity separations proposed for large-scale recovery of saccharide degrading enzymes are limited (Table 1.5). Isolation of ß-galactosidase and glucose isomerase is described for agarose and Sepharose derived matrices. With these relatively expensive matrices ß-galactosidase was recovered from an ammonium sulfate precipitate of Escherichia coli yielding 5 g of enzyme in 2 h in a 1.8 l column [34,35]. The most extensively studied are affinity separations of polysaccharide degrading enzymes using crosslinked substrates.

Affinity separations based on enzyme-substrate interactions

Affinity chromatography on (crosslinked) polysaccharides affords a simple and rapid procedure for the purification of polysaccharide degrading enzymes. This method was described for the isolation and purification of endo-polygalacturonase from Aspergillus niger on crosslinked pectate [36]. Polysaccharides are made insoluble by crosslinking them with an appropriate crosslinking reagent, usually epichlorohydrin. The specificity of the matrices is strongly dependent on the crosslinking conditions [37-39].

This was shown by the fact that pectin esterase isoenzymes and pectate lyases from respectively Citrus sinensis and Bacillus polymyxa could be successfully separated using several matrices with a specific degree of crosslinking [38,39]. This method was further developed for other polysa-

ccharide degrading enzymes like α-amylase, cellulase and pectate lyase using their crosslinked substrates. It appeared possible to isolate α-amylase, endo-polygalacturonase and cellulase from a crude extract of Phoracantha semipunctata in a chromatography system using crosslinked starch, crosslinked pectate and crosslinked cellulase respectively [40]. In Table 1.5 these applications are summarized.

Table 1.5 - Affinity chromatography of (poly)saccharide converting enzymes.

Enzyme	Matrix	References
α-amylase	crosslinked starch	40-43
ß-amylase amyloglucosidase	crosslinked starch	44
endo-polygalacturonase	crosslinked pectate	15,36,37,40,45,46
	crosslinked alginate	15
	alginate beads	21,22,43
	polygalacturonic acid coupled to glass, silica	47,48
pectate lyase pectin esterase	crosslinked pectate, crosslinked alginate	37-39
cellulase	(crosslinked) cellulose	7,40,49-51
ß-galactosidase	PAPTG-agarose[a]	34
	PAPTG-Sepharose[a]	52,53
glucose-isomerase	xylitol on sepharose	54

[a]: *PAPTG = p-aminophenyl-ß-D-thiogalactopyranoside, an inhibitor for the enzyme.*

Other affinity supports were developed by chemical coupling of pectate to inert matrices like porous glass and coated silicas [47,48]. With these supports endo-polygalacturonase was successfully isolated from a culture medium of A. niger and purified by a factor 8.

The most important limitation for large-scale isolation of polysaccharide degrading enzymes on their crosslinked substrates is the potential biodegradability of the matrices. Breakdown of the matrices was observed in several systems [15,38,39,41,49]. In some isolation methods breakdown of the matrix was even used as a means to recover the enzyme [50,51]. There is a need for stable matrices which were developed for endo-polygalacturonase [15,55], ∝-amylase [43], ß-amylase and amyloglucosidase [44].

A general drawback for direct application of such matrices in chromatography is that packed bed columns become fouled and plugged. So removal of solid contaminants from the feed is essential. Furthermore, the throughput makes packed bed chromatography a low productivity system; the processing rate can be a hundred times lower compared with e.g. precipitation methods [56].

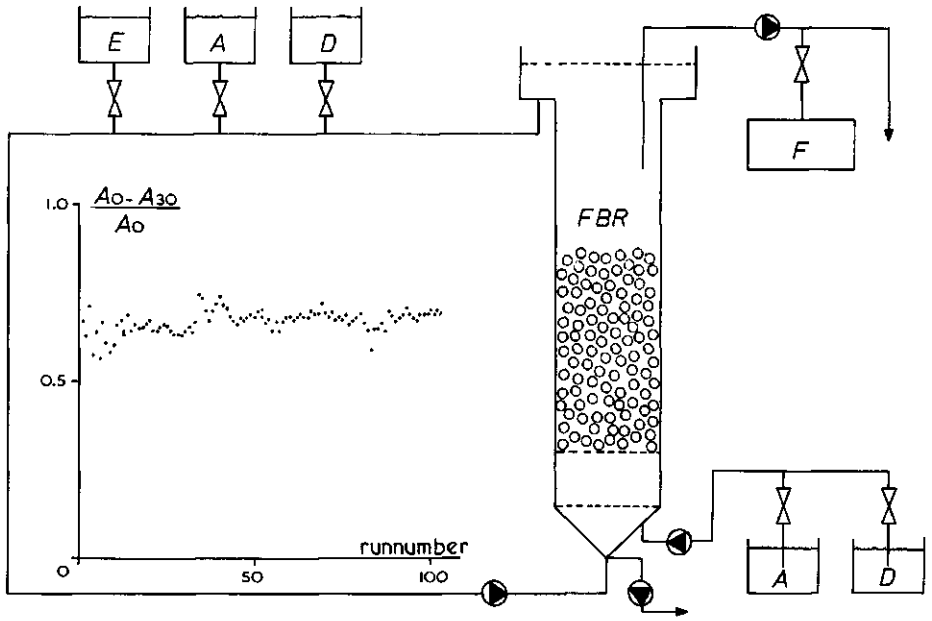
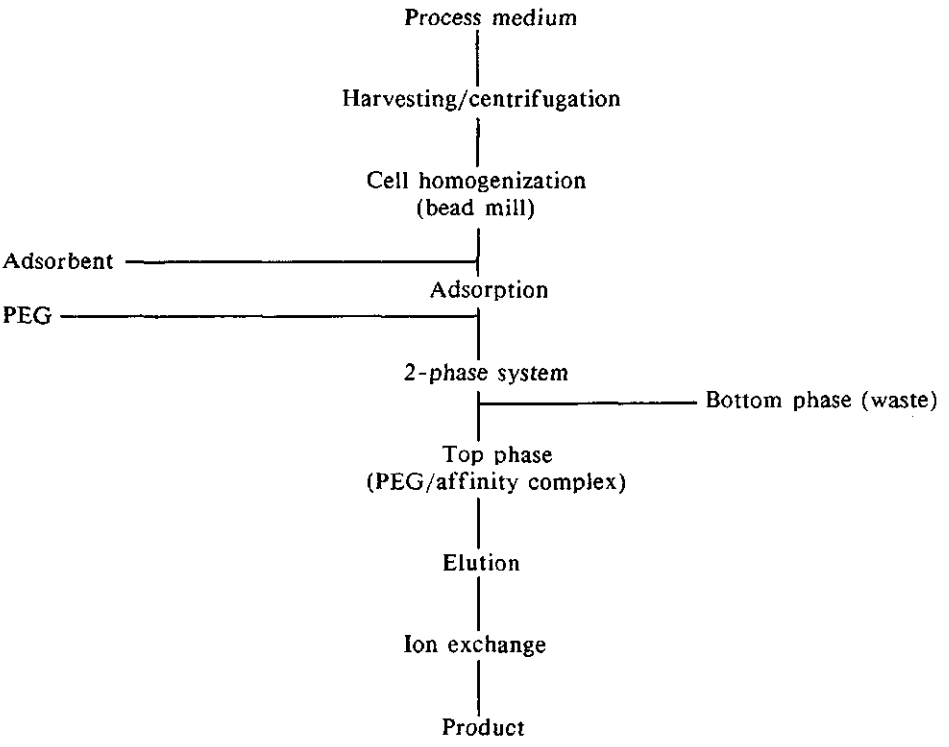


Fig. 1.2 - Repeated adsorption of endo-PG on alginate beads. A, Adsorption medium; D, Desorption medium; E, Enzyme solution; F, Fraction collector; FBR, Fluidized Bed Reactor. Insert: Repeated adsorption end desorption. A_0, enzyme activity in solution before adsorption; A_{30}, enzyme activity remaining in solution after 30 min of adsorption (from ref. [22]).

Alginate, a substrate analogue for pectate, was studied as a matrix for the isolation of endo-polygalacturonase. It appeared possible to isolate the

enzyme from a crude enzyme mixture with this matrix whereas no breakdown was observed [15,22,55]. By converting the alginate into beads by Ca^{2+}-complexation an inert bead was obtained. These beads offer the possibility to isolate endo-polygalacturonase in a fluidized bed reactor and therefore to isolate the enzyme directly from fermentation media. It was shown that this matrix can be used extensively (during hundred runs) to isolate the enzyme from a pectolytic sample without loss of binding capacity (see Fig. 1.2). The enzyme was purified by a factor 3.0, whereas the recovery was complete in this system [22].

For the isolation of ß-amylase and glucoamylase crosslinked glucose homo-oligomers or branched polyglucans were described [44]. By crosslinking reactions of a variety of oligosaccharides or highly branched polyglucans with epichlorohydrin, matrices were obtained that show variation in affinity for ß-amylase and glucoamylase, respectively. The matrices are specific for the two enzymes mentioned and do not bind ∝-amylase from culture filtrates. Applications are proposed in the form of suspension-mixing, packed-bed chromatography and fluidized bed reactors [44].



Scheme 1.4 - Extraction of ß-galactosidase by affinity partitioning.

Affinity partitioning

An interesting development in this field is the application of affinity interactions in a two-phase system. By chemically modifying a ligand so that it partitions exclusively to one phase, a favourable effect on the distribution of the protein will be obtained if it can be bound to the modified ligand [57,58]. ß-Galactosidase was very effectively extracted from a culture medium of E. coli in a PEG/dextran system using phenyl-Sepharose CL-4B as an affinity adsorbent (Scheme 1.4) [29]. The recovery was high, almost no enzyme activity was observed in the liquid phases at equilibrium whereas the affinity complex was partitioned almost completely (99.7%) to the PEG-phase.

Affinity cross-flow filtration

This method of isolation combines the properties of affinity interactions and membrane separations [56,59]. The protein to be purified binds to an affinity ligand which is circulated through a circuit containing at least two membrane units. Impurities and unbound proteins can pass through the membrane, whereas the affinity ligand and the protein-ligand complex can not. In the first membrane unit the product is specifically adsorbed by the adsorbent whereas the impurities are discarded from the system. In the next membrane unit the product is desorbed and separated from the matrix by membrane filtration. The adsorbent is reconditioned and recirculated to the adsorption module. For a successful application the membrane should have a high hydraulic permeability for water, a sharp molecular weight cut-off, a good mechanical stability, a high fouling resistance and it should be easy to clean and reusable. Membranes which meet these demands are now available [56]. A process diagram for an affinity cross-flow filtration is given in Fig. 1.3.

This system was used for the recovery of several enzymes (reviewed in ref. [56]). ß-Galactosidase was purified in a continuous affinity recycle extraction [23]. As the affinity adsorbent commercially available p-aminophenyl-ß-D-thiogalactopyranoside (PABTG-) agarose beads were used. These sorbents are circulated through two membrane units allowing the enzyme to be purified from clarified enzyme solutions as well as from media with cell debris. The enzyme was both purified and concentrated 8.9-fold in this process, the recovery yield being 90%. With a crude cell homogenate of E. coli a purification factor of 31.3 was achieved from a protein solution of 0.5 $mg.ml^{-1}$. Recovery however was lowered to 70%. The system appeared to be stable during 6 h under steady state conditions [23].

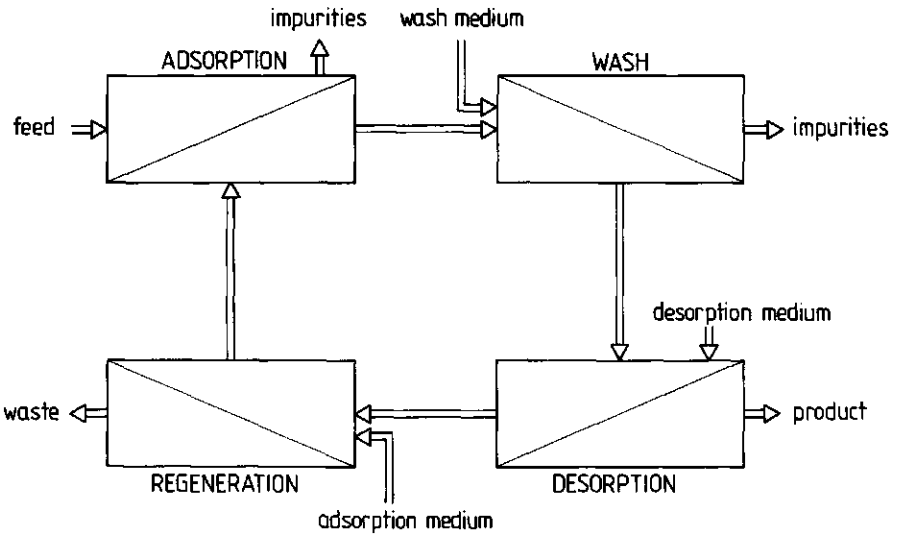


Fig. 1.3 - Flowsheet for affinity ultrafiltration. The affinity adsorbent is circulated through the system.

1.5 SCOPE AND OUTLINE OF THE THESIS

The bulk enzymes that are on the market are only partly purified. Their recovery processes from media show a series of rather straightforward processes with low selectivity. The increasing demand for more purified low cost bulk enzymes that can be expected, requires new separation techniques that combine selectivity with rather simple process design. Nowadays a purification process for bulk enzymes consists of a number of process steps (f.e. filtration, concentration, precipitation) which can be avoided and replaced by a one step purification process resulting in reduction of purification costs and yielding more purified products. Many techniques, which are considered to be important in current downstream processing, can also be applied in the field of relatively cheap bulk enzymes such as polysaccharide degrading ones. As to the phase in the downstream processing where these isolation methods can be successfully introduced, one is referred to Table 1.4.

In this study the development and application of cheap affinity-adsorbents, needed for the recovery of endo-polygalacturonase and α-amylase,

are described. These matrices can in principle be applied directly to a process medium allowing a quick and selective extraction of the enzymes from a process medium. In this way a number of process steps which are now in use to purify these enzymes can be replaced by a direct extraction of the enzymes from the fermentation media in a few steps. Important here is the consideration that the enzymes involved are mostly low cost products which therefore do not allow for expensive separation techniques. Therefore cheap and easy to prepare affinity adsorbents have to be developed for the enzymes.

Chapter 2 deals with the development of alginate beads as an affinity adsorbent for endo-polygalacturonase. The application of the adsorbent is described. In Chapter 3 mass transfer in this system is studied.

Chapter 4 deals with the developments of an affinity adsorbent for α-amylase and the optimization of the process conditions. Chapter 5 deals with the application of the adsorbent in a continuous purification process. In Chapter 6 the development of affinity adsorbents for α-amylase which can be used in fluid bed separations are described. Finally, a general discussion is given in Chapter 7.

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CHAPTER 2

ISOLATION AND PURIFICATION OF ENDO-POLYGALACTURONASE BY AFFINITY INTERACTIONS IN A FLUIDIZED BED REACTOR

SUMMARY

Endo-polygalacturonase can be purified and isolated from an industrial pectolytic enzyme preparation using alginate as an affinity adsorbent in a fluidized bed reactor. Alginate is a substrate analogue for the natural substrate pectate. It was transformed into beads by Ca^{2+}-complexation.

In this work the adsorption and desorption kinetics of the system are studied. First the adsorption and desorption conditions were optimized with respect to pH, calcium chloride concentration and ionic strength of the buffers used. The adsorption reaction is governed by the diffusion of the enzyme into the beads and by the loading capacity of the beads, and the adsorption process has been described by means of a shrinking core model commonly used for the description of adsorption processes. The diffusion velocity of the enzyme in alginate beads was determined. The desorption process is regulated by the diffusion velocity of the enzyme and could be described using a simple diffusion model.

The stability of the beads was tested in repeated adsorption/desorption experiments in a batch fluidized bed reactor isolating the enzyme from an industrial pectolytic sample. The adsorption capacity of the beads for the enzyme did not decrease for at least 100 cycles suggesting that the beads were stable during the process.

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2.1 INTRODUCTION

One of the current areas of interest in downstream processing is the application of affinity separation techniques to the large-scale purification of proteins [1-4]. Affinity separations are based on the specific interactions between biological molecules and a specific ligand for these molecules. This offers the possibility of isolating the protein from a diverse mixture of molecules even in cases where its concentration is low.

It may be useful to introduce an affinity adsorption step at an early stage in the purification process, when it can replace a number of commonly used separation procedures such as centrifugation, filtration and ultrafiltration and permit direct isolation of the protein. Problems related to the introduction of packed-bed chromatography at an early stage of the isolation process are clogging and fouling of the column resulting in pressure build-up and poor performance [1]. This problem can be avoided by designing an affinity adsorbent which is sufficiently large and dense to be used in a fluidized bed reactor [5].

Affinity chromatography is a well-known procedure for purifying endo-polygalacturonase (endo-PG). Crosslinked pectate was the first adsorbent to be used for purifying the enzyme [6,7]. However, the disadvantage was that the pectate was hydrolyzed to some extent during the purification process [6]. Alginate, a substrate analogue for pectate, can also be crosslinked with epichlorohydrin. In this form it has proved to be a useful adsorbent for endo-PG in a packed-bed chromatographic column [7]. To apply this technique in a fluidized bed reactor the alginate was transformed into particles by preparing the Ca^{2+}-complex. The adsorption characteristics of the alginate - endo-PG system were investigated under different reaction conditions (i.e. pH, calcium chloride concentration and ionic strength) with respect to the equilibrium state [8].

In this study the suitability of the alginate particles for the isolation of endo-PG in repeated batch fluidized bed isolation processes was investigated as a first step in the development of a continuous process for the isolation of the enzyme from a fermentation broth. The binding characteristics of the adsorbent were investigated using a commercial enzyme sample (Rapidase, Gist brocades, Seclin, France). The adsorption and desorption kinetics were studied and described with a model, and the adsorbent was used in a continuous adsorption/desorption process to analyse the stability and the binding capacity.

2.2 THEORY

Adsorption model

The following assumptions are made when using a shrinking core model:

a/ The rate of protein adsorption is much higher than the diffusion velocity (i.e. the adsorption is at equilibrium and the system is fully diffusion controlled).

b/ The diffusion coefficient is constant during the experiment.

c/ The protein adsorption capacity of the support is much larger than the amount of protein dissolved in the pores of the support (i.e. the solute held in the pores is negligible).

d/ The protein is irreversibly bound to the support.

e/ The isotherm governing the loading process is of the saturation type (i.e. for $C > 0$, $q = q_{\infty}$ and for $q < q_{\infty}$, $C = 0$).

Infinite bath experiment

For any experiment with a constant enzyme concentration in the liquid phase (infinite bath experiment) the following equation for the loading of the particle with enzyme has been derived [9]:

$$\frac{-D_{eff} \times C(0)}{q_{\infty} \times \rho_p} dt = \frac{(R - r_b)\, r_b}{R} dr_b \qquad (1)$$

The following parameters are defined:

$$t_{\infty} = \frac{q_{\infty} \times \rho_p \times R^2}{6 D_{eff} \times C(0)} \qquad (2a)$$

$$Ra = \frac{r_b}{R} \qquad (2b)$$

$$t_i = \frac{t}{t_{\infty}} \qquad (2c)$$

Upon integration with respect to time this yields

$$t_i = 1 - 3(Ra)^2 + 2(Ra)^3 \quad (3a)$$

or

$$t_i = 3 - 3\,(1-B)^{2/3} - 2B \quad (3b)$$

where

$$B = q(t)/q_\infty \quad (3c)$$

When the diffusion coefficient (D_{eff}) and the loading capacity (q_∞) are known the equilibrium time (t_∞) can be calculated from eqn. (2a). Using eqns. (3a)-(3c) the penetration depth of the enzyme and the time needed to attain the average loading of the particle $q(t)/q_\infty$ can be found. For $t_i = 1$, $q(t)/q_\infty = 1$.

Finite bath experiment

When spherical beads (radius R) free of enzyme are suspended in a finite solution with an initial enzyme concentration C(0) and the enzyme diffuses into the beads and is irreversibly adsorbed, then the bulk liquid concentration C(t) is given by the following equation [10-12]:

$$\frac{D_{eff} \times (1-A) \times t}{R^2 \times A} = -\frac{1}{3}\ln\left(\frac{a^3 + w^3}{a^3 + 1}\right) + \frac{1}{6a}\ln\left\{\frac{(a^2 - a + 1)(a + w)^2}{(a^2 - aw + w^2)(a + 1)^2}\right\}$$

$$+ \frac{1}{a \times 3^{1/2}}\tan^{-1}\left(\frac{2 - a}{a \times 3^{1/2}}\right) - \tan^{-1}\left(\frac{2w - a}{a \times 3^{1/2}}\right) \quad (4)$$

where

$$G = \frac{A \times C(0)}{q_\infty \times \rho_b} \quad (5a)$$

and

$$A = \frac{V_l}{V_l + V_s} \quad (5b)$$

and

$$a = (G-1)^{1/3} \qquad G > 1 \tag{6a}$$

$$a = 0 \qquad G = 1 \tag{6b}$$

$$a = -(1-G)^{1/3} \qquad G < 1 \tag{6c}$$

and

$$w = [1-G\{1-C(t)/C(0)\}]^{1/3} \tag{7a}$$

$$= [1-q(t)/q_{\infty}]^{1/3} \tag{7b}$$

Desorption

The desorption reaction rate is assumed to be controlled by the diffusion of the enzyme out of the beads. When the beads are in equilibrium with the liquid phase during the adsorption reaction, then the enzyme is assumed to be homogeneously spread throughout the beads. The desorption rate is then given by the following equation for diffusion of substrates out of a sphere [13,14]:

$$C(t) = \frac{C_p(0)}{(1+\alpha)} \left\{1 - \sum_{n=1}^{\infty} \frac{6\alpha(1+\alpha)\exp(-D_{eff}x_n^2 t/R^2)}{9 + 9\alpha + x_n^2\alpha^2}\right\} \tag{8a}$$

or

$$\frac{C(t)}{C_l(\infty)} = 1 - \sum_{n=1}^{\infty} \left\{\frac{6\alpha(1+\alpha)\exp(-D_{eff}x_n^2 t/R^2)}{9 + 9\alpha + x_n^2\alpha^2}\right\} \tag{8b}$$

where x_n represents the non-zero positive roots of

$$\tan x_n = \frac{3x_n}{3 + \alpha x_n^2} \tag{9}$$

$$\alpha = \frac{3V_l}{n \times 4\pi R^3} \tag{10}$$

where R is the bead diameter, n, the number of beads and V_l, volume of the

liquid phase, and $C_l(\infty)$ (the equilibrium concentration of the enzyme in the liquid phase) is related to $C_p(0)$ by

$$C_l(\infty) = \frac{C_p(0)}{(1 + \alpha)} \qquad (11)$$

2.3 MATERIALS AND METHODS

Sodium alginate (Manucol DM) is a product of Alginate Industries (London); Rapidase C-80 is an enzyme preparation of Rapidase (Gist brocades, Seclin, France). For further specifications of these products see ref. [8].

Determination of enzyme activity and protein content

Polygalacturonase activity was determined by a modified ferricyanide test [15] or by an automated neocuproin test [16] using an auto analyser (Skalar) as described in ref. [8].

Protein content was determined by using the method of Lowry et al. [17].

Preparation of alginate beads

20 g sodium alginate (Manucol DM) was dissolved in distilled water making up a total volume of 1000 ml (2% w/v). The alginate was made to dissolve by using an ultraturrax mixer for 5 min.

Beads were prepared by dropping this solution through a dropping funnel into a continuously stirred 200 mM calcium chloride solution. The beads obtained were stirred for 24 h in a fresh 200 mM calcium chloride soultion and then stored in a 30 mM calcium chloride solution at 4 ^{0}C.

Large quantities of beads were prepared using a resonance nozzle [18]. 20 litre of alginate could be processed by this technique in one hour.

Determination of bead diameter

The bead diameter was determined by using a binocular microscope (Zeiss-Ikon) with a measuring device. The bead diameter used in the calculations was the average of 20-30 values of bead diameters measured in this way. The standard deviation was less than 4% of the total diameter in all experiments.

Adsorption isotherms

Adsorption isotherms were determined by incubating 0.5 g alginate beads

(R = 0.075 cm, ρ_p = 1.006 g/ml) with varying amounts of Rapidase C-80 (0 - 540 U/ml) in 25 ml 100 mM sodium acetate buffer pH 3.8 with 10 mM calcium chloride for 48 h. One Unit (U) is defined as the amount of enzyme that liberates 1 μmol of reducing end groups from polygalacturonic acid (Sigma) in 1 min, T = 30 ^0_cC, pH = 4.2. The amount of enzyme adsorbed was found by measuring the decrease of enzyme activity in the liquid phase or by desorption of the enzyme from the beads. For this desorption 20 beads (0.035 ml) were suspended in 1 ml 100 mM sodium acetate buffer of pH 3.8 with 10 mM calcium chloride and 1 M sodium chloride. After 24 h the enzyme activity in the liquid phase was determined.

Adsorption kinetics

a. Constant liquid enzyme concentration (infinite bath experiment)

0.5 g of alginate beads (R = 0.075 cm) were suspended in 200 ml of a solution of Rapidase C-80 (360 U/ml) in 100 mM sodium acetate of pH 3.8 also containing 10 mM calcium chloride. At chosen time intervals about 50 beads were taken out of the bath using a sieve. The beads were washed with the adsorption buffer and dried with a tissue. Then two portions of 20 beads were each suspended in 1 ml desorption buffer (i.e. 100 mM sodium acetate, pH 3.8, with 10 mM calcium chloride and 1 M sodium chloride) and desorbed for 24 h. The amount of enzyme previously bound to the particles was calculated from the amount of enzyme released by desorption assuming the enzyme to be totally desorbed.

b. Batch adsorption

Adsorption kinetics were determined by incubating different quantities of alginate beads (1 - 4 g, R = 0.075 cm) in 20 ml Rapidase C-80. In most experiments the starting enzyme concentration was 360 U/ml. Samples (100 μl) were taken during a time span of 120 min. At equilibrium another sample was taken. The decrease in the volume of the liquid during the experiment was less than 5% of the total volume. The data were corrected for this change in volume. The adsorption medium was a 100 mM sodium acetate, 10 mM calcium chloride buffer of pH 3.8.

Desorption kinetics

Alginate beads loaded with enzyme were suspended in the desorption buffer (i.e. 100 mM sodium acetate, 10 mM calcium chloride, 1 M sodium chloride, pH 3.8). The desorption reaction was followed by taking samples

during a period of 120 min. In these experiments the change in the liquid volume was less then 5%. The data were corrected for this change in volume.

Determination of the diffusion coefficient of endo-polygalacturonase

Alginate beads loaded with non-adsorbed enzyme were prepared as follows. A Rapidase solution (90 U/ml) in 100 mM sodium acetate, pH 6.0, was prepared. Different amounts of alginate were added to defined portions of this solution in a range from 0.5 to 3% (w/v). The alginate was made to dissolve by stirring for 5 min with an ultraturrax stirrer. The enzyme/alginate solutions obtained were deaerated in a desiccator and beads were prepared by passing the solutions through a dropping funnel into a Rapidase solution (90 U/ml) in 100 mM sodium acetate buffer, pH 6.0, with 200 mM calcium chloride. The beads were desorbed as described above.

Fluidized bed reactor

Experiments to test the beads in a repeated adsorption/desorption process were conducted in a fluidized bed reactor (V = 450 ml, d = 3.4 cm, h = 50 cm). The reactor contained 100 g of alginate beads. The beads were fluidized by means of a recirculation pump (Watson-Marlow) with silicone tubing. The fluidisation velocity was 0.11 cm/s. The beads were loaded for 30 min in an enzyme solution (38 U/ml, 350 ml). Then the adsorption medium was pumped out of the column. Subsequently the beads were washed with 350 ml of the adsorption buffer. The enzyme was desorbed by introducing the same volume of desorption buffer (350 ml). After desorption (30 min) the column was regenerated by replacement of the liquid with the adsorption buffer to remove the excess of salt.

2.4 RESULTS

Optimization of the adsorption and desorption conditions

Adsorption

Several factors affect the binding velocity of endo-PG to alginate beads. The pH and calcium chloride concentration, together with the ionic strength of the adsorption medium, are the most important factors [8,19]. The dependence of the adsorption characteristics on the pH is observed in the adsorption isotherms for the alginate/endo-PG system [8]. The influence of pH on adsorption is shown in Fig. 2.1(a). The apparent adsorption at pH 6.0 can be

explained by diffusion of the enzyme into the beads. This can be shown by calculating the enzyme concentration in solution when equilibrium is reached. This concentration should be $C(0) \times V_l/(V_s + V_l)$, where V_l is the volume of the liquid phase, V_s the volume of the solid phase and C(0) the starting concentration of the enzyme in the liquid phase. The adsorption increases with decreasing pH. A pH of 3.8 was chosen for the adsorption experiments, since below pH 3.8 loss of enzyme activity occurs owing to inactivation ref. [8].

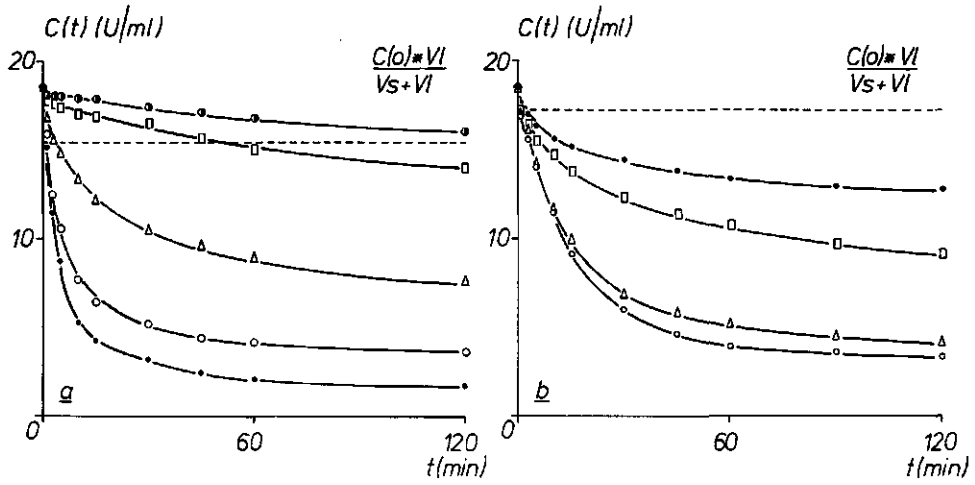

Fig. 2.1 - (a)Binding of endo-PG to alginate as a function of pH.
●, pH 3.0; ○, pH 3.8; △, pH 4.2; □, pH 5.0; ◑, pH 6.0. Buffer: 100 mM sodium acetate, 10 mM calcium chloride. Amount of alginate beads: 20 ml, R = 0.075 cm. Liquid volume: 100 ml.
(b)Binding of endo-PG to alginate at different calcium chloride concentrations. Buffer: 100 mM sodium acetate, pH 3.8. Amount of alginate beads: 15 ml, R = 0.075 cm. Liquid volume: 200 ml.
○, 5 mM calcium chloride; △, 10 mM calcium chloride;
□, 30 mM calcium chloride; ●, 50 mM calcium chloride.

The negative influence of the calcium chloride concentration on the adsorption is shown in Fig. 2.1(b). It is necessary to maintain a low concentration of calcium chloride in solution to stabilize the beads. Without calcium chloride in the adsorption and desorption buffers, alginate is washed out of the beads. At 10 mM calcium chloride no alginate wash-out occurs, the reaction velocity is influenced only in a limited way and the loading capacity

of the alginate for the enzyme is acceptable [8]. For the adsorption buffer 100 mM sodium acetate, 10 mM calcium chloride and a pH of 3.8 were used in all further experiments.

Desorption

It is known that the enzyme can be eluted from the alginate by applying a buffer with a pH of 5.8-6.0 or by applying a buffer with high ionic strength (e.g. 1 M sodium chloride) [6,7]. It was found that the pH was not important when a buffer of high ionic strength was applied. Furthermore elution of the enzyme was found to be equally effective when a buffer with 0.5 M sodium chloride was used instead of 1 M sodium chloride. At sodium chloride concentrations lower than 0.5 M the desorption reaction rate was reduced and the enzyme was not completely released from the adsorbent. A 100 mM sodium acetate, 10 mM calcium chloride buffer of pH 3.8 with 1.0 M sodium chloride was used in the desorption experiments, since this allowed a constant pH to be maintained during the whole process.

Adsorption isotherm

The adsorption isotherm for the endo-PG/alginate system is shown in Fig. 2.2. Under the experimental conditions chosen the beads could be loaded to

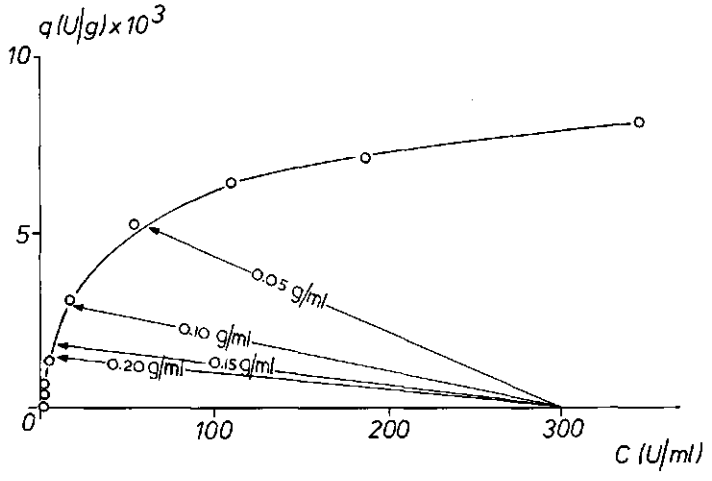

Fig. 2.2 - Adsorption isotherm of the endo-polygalacturonase/ alginate system. The values in g/ml refer to alginate concentrations used in the finite bath experiments (see Fig. 2.5).

8000 U/g, which represents 0.28 mg of enzyme per mg of alginate (specific activity of endo-PG is 580 U/mg, alginate beads contain 5% w/v of alginate [8]). A part of the enzyme activity present in the Rapidase sample can not be absorbed by the alginate [8]. This amount was determined to be 16% of the total enzyme activity in the sample. The enzyme concentrations were corrected for this fraction.

Determination of diffusion coefficients of endo-PG

To determine the effective diffusion coefficient (D_{eff}) of endo-PG in alginate beads, beads with different alginate concentrations and loaded with non-adsorbed enzyme were prepared. Below an alginate concentration of 0.5% (w/v) it is impossible to prepare rigid beads. The bead diameter was determined and the beads were suspended in the desorption buffer. The diffusion of the enzyme out of the beads was followed by measuring the enzyme activity, released into the desorption buffer, as a function of time. The data were fitted with eqn. (8) to determine the diffusion coefficient D_{eff}. Figure 2.3(a) shows the experimentally determined data for varying alginate concentrations in the beads and the diffusion curves calculated.

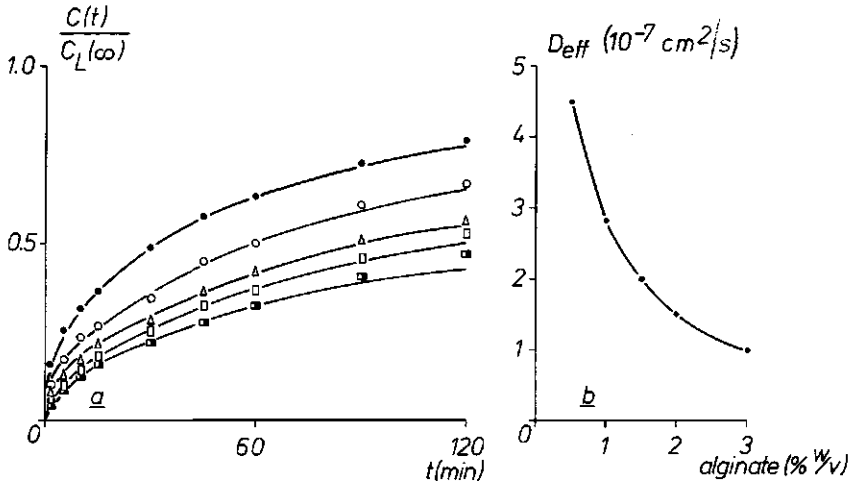

Fig. 2.3 - (a) Measured and calculated concentration values of endo-PG released from alginate beads. Amount of alginate: 20 ml. Buffer: 200 ml 100 mM sodium acetate; 1.0 M sodium chloride; 10 mM calcium chloride. pH 3.8; ● *,0.5% (w/v) beads. R = 0.185 cm;* ○ *,1.0% (w/v) beads. R = 0.201 cm;* △ *,1.5% (w/v) beads. R = 0.214 cm;* □ *,2.0% (w/v) beads. R = 0.213 cm;* ◨ *,3.0% (w/v) beads. R = 0.210 cm.*
(b) Diffusion coefficient as a function of alginate concentration in the beads, for the curves given in Fig. 2.3(a).

The diffusion velocity decreases with increasing alginate concentration in the beads (Fig. 2.3(b)). The values obtained are in good agreement with the diffusion coefficients found by other authors for the diffusion of proteins in alginate beads [14]. They are smaller than the values usually obtained for proteins with a molecular weight of approximately 40,000 D in water, and which vary in general between 5 and 6 $\times$ 10^{-7} cm^2/s for fibrillar proteins and 7 and 9 $\times$ 10^{-7} cm^2/s for globular proteins [20].

Adsorption kinetics

Infinite bath experiment

When the experiment on the adsorption of endo-PG to alginate beads was conducted using the infinite bath method the enzyme concentration in the liquid phase is changed by less than 5% during the experiment. This was achieved by introducing 0.5 g of beads into 200 ml of enzyme solution (360 U/ml). Figure 2.4 shows the relative loading ($q(t)/q_\infty$) as a function of time for three different bead diameters (0.075 cm, 0.119 cm and 0.245 cm).

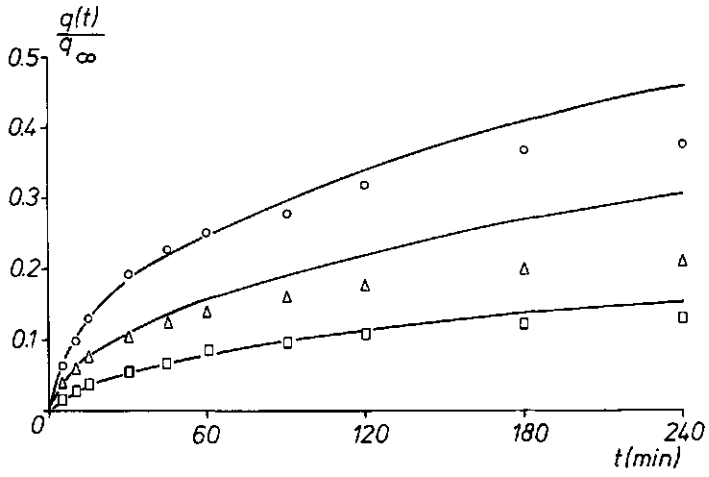

Fig. 2.4 - Adsorption of endo-PG to alginate beads in an infinite bath experiment. Buffer: 100 mM sodium acetate; 10 mM calcium chloride pH 3.8. Enzyme concentration: 300 U/ml. Alginate beads: ○ *, R = 0.075 cm;* △ *, R = 0.119 cm;* □ *, R = 0.245 cm;* ——*, Model:* D_{eff} *= 1.5 $\times$* 10^{-7} cm^2/s*,* q_∞ *= 7700 U/g.*

Using eqns. (1) and (2) the adsorption and the loading of the beads were modelled by introducing the experimentally found loading capacity (q_{∞}) of 7700 U/g determined from the adsorption isotherm and the apparent diffusion coefficient (D_{eff}) of 1.5 × 10^{-7} cm^2/s found as described in the previous section.

Figure 2.4 shows that at the start of the experiment the loading of the beads is predicted very well with the separately determined values for q_{∞} and D_{eff}. After 180 min the adsorption of enzyme by the beads is slower than predicted by the model, possibly because of decreasing diffusivity of the enzyme in the loaded beads or fouling of the bead surface.

It may be concluded from the infinite bath experiments that the adsorption of the enzyme by the beads can be described reasonably well by the shrinking core model.

Finite bath experiments

The binding kinetics of endo-PG in adsorption experiments with changing bath concentrations was also investigated with the aid of the shrinking core model. The adsorption isotherm of the endo-PG/alginate system is given in Fig. 2.2. The lines designated in Fig. 2.2 for the different alginate concentrations relate the decrease in enzyme concentration in the liquid phase to the average loading of the beads in the various experiments. The slope of each line is given by $q/(C - C(0))$.

In Fig. 2.5 the decrease in enzyme activity in the liquid phase is shown as a function of time at a single starting concentration of enzyme (C(0) = 300 U/ml) and different alginate concentrations. The kinetics were described using the shrinking core model. Equation (4) gives the analytical solution for this experiment. The model was used with the loading capacity as the fitting parameter and a fixed diffusion coefficient of 1.5 × 10^{-7} cm^2/s as determined before. The results are collected in Table 2.1.

The values of q_{∞} are between the maximum value (q_{∞} = 7700 U/g, i.e. the equilibrium value for C(0) = 300 U/ml) and the equilibrium values for each case (Fig. 2.2). Figure 2.5 shows the fitting of the curve for the adsorption process using the values from Table 2.1. It is clear from Table 2.1 that at low alginate concentrations the shrinking core model is at its most acccurate. This can be explained by the fact that in this case, during the adsorption time considered, the loading of the beads is determined by that part of the isotherm which is almost a 'saturation' type isotherm (see Fig. 2.2). The shrinking core model appears to be sufficiently accurate in predicting the binding of the

enzyme to the beads and can be used in calculating the performance of the adsorption process in a fluidized bed reactor.

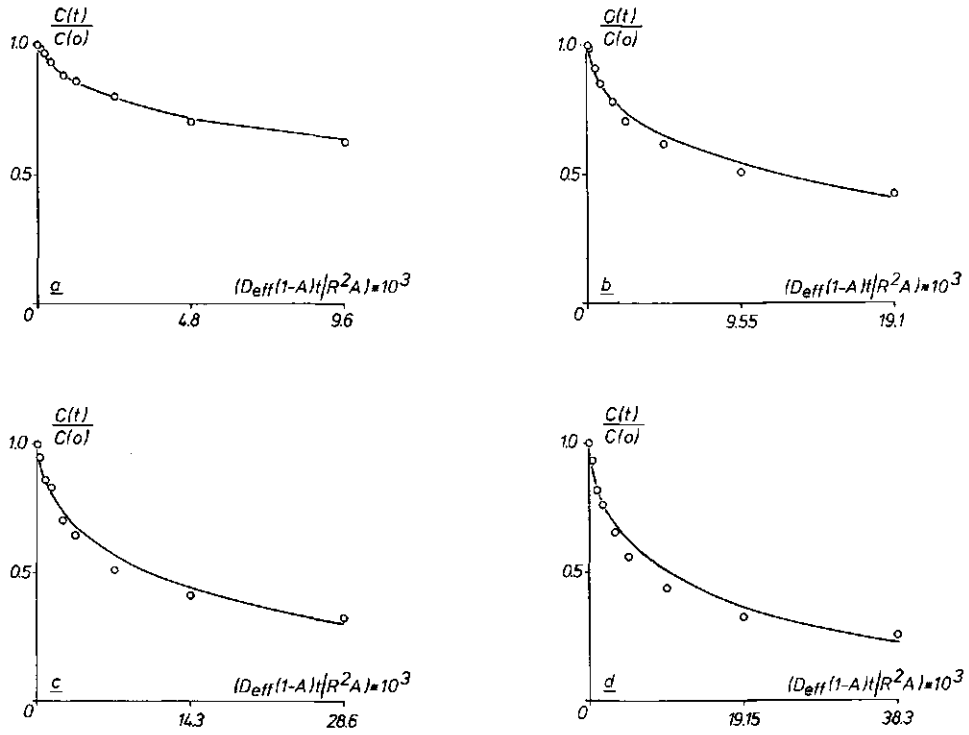

Fig. 2.5 - Adsorption of endo-PG into alginate beads. Curve-fits according to the shrinking core model. Experimental conditions: see text. Parameters in the model as in Table 2.1. Alginate concentrations: a: 0.05 g/ml; b: 0.10 g/ml; c: 0.15 g/ml; d: 0.20 g/ml.

Table 2.1 - Shrinking core model - calculated loading capacities (q_∞).

concentration of alginate ($g \times ml^{-1}$)	q_∞ (calculated) ($U \times g^{-1}$)	D_{eff} (experimental) (10^{-7} cm^2/s)
0.05	7020	1.5
0.10	5850	1.5
0.15	4350	1.5
0.20	3540	1.5

Desorption kinetics

Desorption of the enzyme can be described by assuming that the diffusion of the enzyme is the limiting factor in the desorption process. Figure 2.6 shows the desorption curves for beads loaded during different adsorption times. The desorption reaction is slowed down with a prolonged adsorption time even when no further enzyme is bound from the liquid phase (Table 2.2). This may be due to migration of the enzyme inside the beads in case of prolonged adsorption times.

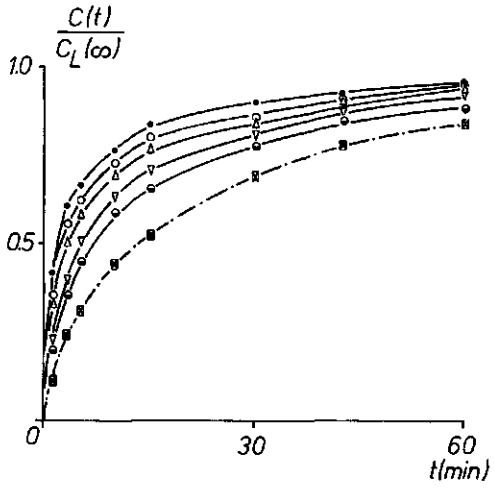

Fig. 2.6 - Desorption profiles of endo-PG from alginate beads after different adsorption times. Amount of alginate: 20 g, R = 0.078 cm. Desorption buffer: 100 mM sodium acetate, 10 mM calcium chloride, 1.0 M sodium chloride, pH 3.8. Liquid volume: 150 ml. Adsorption time: ● , 15 min; ○ , 30 min; △ , 60 min; ▽ , 120 min; ◒ , 180 min; □ , 24 h; X , 48 h. —•—•— , Calculated diffusion curve, D_{eff} = 1.8 × 10^{-7} cm^2/s (curves 24 h and 48 h).

After 24 hours of adsorption the desorption pattern becomes constant. The diffusion coefficient describing the desorption reaction is then 1.8 × 10^{-7} cm^2/s. This value is in good agreement with the results found for the particles loaded with non adsorbed enzyme.

Repeated adsorption/desorption experiments

Repeated adsorption and desorption experiments were conducted in a batch fluidized bed reactor (Fig. 2.7). During every tenth run the kinetics of

Table 2.2 - Adsorption of endo-PG on alginate beads as a function of adsorption time[a].

C(0) (U)	adsorption reaction time (min)	adsorbed amount (U)	bead loading (U/g)
4880	15	2220	111
4880	30	2750	137
4880	60	3100	155
4880	120	3220	161
4880	180	3530	176
4880	1440	3420	171
4880	2880	3530	177

[a] *R = 0.075 cm; C(0) = 18 U/ml; Alginate concentration : 0.2 g/ml.*

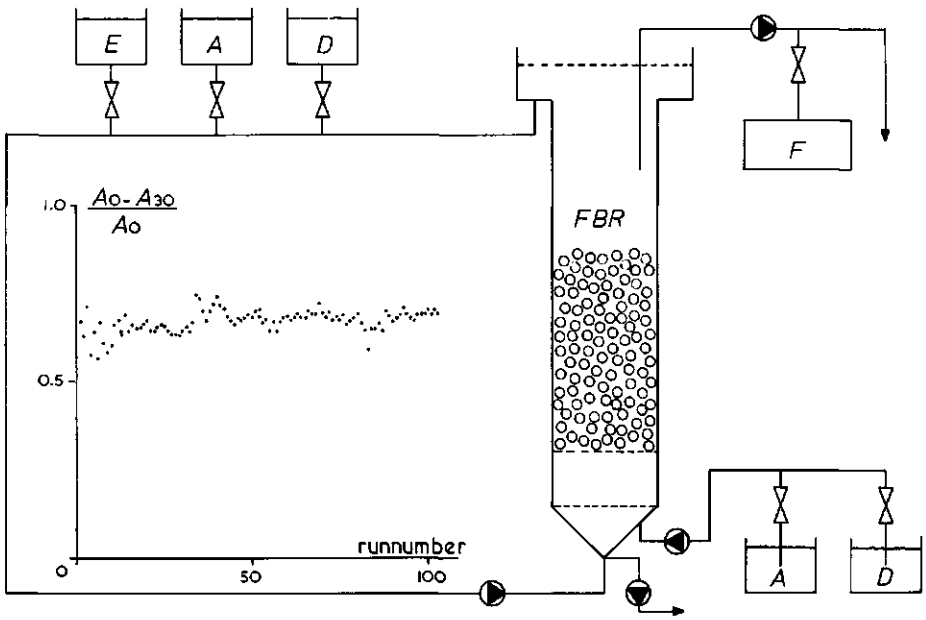

Fig. 2.7 - Fluidized bed reactor. A: Adsorption buffer reservoir; D = Desorption buffer reservoir; E = Enzyme solution reservoir; FBR = Fluidized bed reactor; F = Fraction collector; ◑ = Pump.
Insert: Adsorption of endo-PG to alginate beads in a continuous adsorption/desorption experiment. Experimental details: see text. A_0 = Enzyme activity in enzyme solution before the adsorption step, A_{30} = Enzyme activity remaining in solution after 30 min of adsorption.

the binding process was followed by collecting samples (V = 1 ml) and assaying them for enzyme activity.

Figure 2.7 (insert) shows the amount of adsorption of the enzyme activity in 30 min over 100 cycles. It is clear that the adsorption capacity does not change under the reaction conditions chosen. The results from these experiments are collected in Table 2.3. The velocity of the adsorption reaction in the reactor appeared to be constant during the hundred runs. No slowing down of the reaction velocity was observed.

Table 2.3 - Repeated adsorption and desorption of endo-PG on alginate beads- Enzyme activity and protein content of the eluted fractions.

	Enzyme activity (n=100)		Protein content (n=40)		Specific activity (n=40)
	(U/ml)	(%)	(mg/ml)	(%)	(U/mg)
Enzyme solution	38.0	100	0.93	100	40.8±1.5
Adsorption (30 min)	12.5	32.8±3.3	0.58	62.4±2.9	21.6±2.1
Wash step	3.9	10.1±1.3	0.12	12.9±2.0	32.5±3.9
Desorption	21.1	55.6±5.1	0.17	18.3±1.8	124.1±10.1
Regeneration step 1	3.0	7.9±1.3	0.04	4.2±1.8	75.0±12.1
step 2	20.4	1.0±0.5	0.016	1.7±0.7	25.0±11.2
Total recovery		107.4±5.3		99.5±4.4	

In the desorption step an enriched enzyme fraction is obtained and purified by a factor 3 with respect to the original Rapidase solution. The protein balance gives a recovery of 99.5%, so almost all the injected material is recovered during the process. The enzyme activity balance gives a recovery of 107% of the injected enzyme activity. Somewhere during the process there appears to be an activation of the enzyme, which may be caused by removal of inhibitory compounds in the crude preparation. It must be noted also that some wash-out of the bound enzyme activity occurs in the wash step. This amount is estimated to be 7% of the originally bound enzyme activity, taking the specific activity of the adsorbed fraction and the remaining fraction after adsorption as the parameters for calculation.

2.5 DISCUSSION AND CONCLUSIONS

The adsorption and desorption mechanisms of the binding of endo-polygalacturonase to alginate beads are described. It appears possible to isolate and purify the enzyme using the beads in a repeated batch process. The adsorption conditions have been optimized. The most important parameters are pH, calcium chloride concentration and the ionic strength of the media used. pH has a large effect on the adsorption equilibrium [8] and calcium chloride has to be present to stabilize the beads. Reaction velocity and loading capacity are acceptable with concentrations of calcium chloride up to 10 mM. At higher concentrations there is a rapid decrease in binding capacity [8]. From adsorption studies with an adsorption buffer using increasing concentrations of sodium chloride, it appears that at concentrations of 100 mM or above adsorption is hindered [8].

Prediction of the binding kinetics was done with a shrinking core model. The adsorbing enzyme fraction of the Rapidase solution can be considered as a one-component system. It was found that 90-95% of the binding enzyme activity consists of one endo-PG with a molecular weight of 38,000 D (specific activity 580 U/mg). It was decided therefore to describe the adsorption reaction with a model for a single component. The most important parameters in the model are the quantity of alginate and the radius of the beads which could be measured directly, together with the diffusion velocity of the enzyme in the beads and the loading capacity of the adsorbent for the enzyme, which were determined experimentally. It appeared that prediction of the binding kinetics could be done quite accurately with this model. Some problems were encountered in the experiments with changing bath concentrations, probably

because of the shape of the adsorption isotherm of the endo-PG/alginate system. In the model a saturation-type isotherm is assumed, so the binding capacity of the adsorbent is thought to be independent of the bulk and pore liquid concentration. As can be seen from the isotherm (Fig. 2.2) this assumption is not valid for the system described here. What can be expected then is that the value for the loading capacity of the adsorbent q_{∞} obtained from the model will be between the values for q_{∞} at the start of the experiment and the value at equilibrium. This was confirmed by calculations with a diffusion coefficient of 1.5×10^{-7} cm^2/s. At low alginate concentrations the adsorption reaction was described accurately using the experimentally determined loading capacity and diffusion coefficient. From the adsorption isotherm it can be seen that the maximum capacity of the support is approximately 7700 U/g adsorbent. On a dry weight basis this means that 265 mg enzyme binds to one g alginate (so one mole of enzyme binds to approximately 800 moles of uronic acid residues).

The optimum pH for binding is 3.8 [8]. In terms of the shrinking core model used, the higher reaction velocity can be explained by assuming the loading capacity to be higher at lower pH. The differences in reaction velocity for different reaction conditions (i.e. ionic strength, pH) can also be interpreted in terms of the model as a difference in loading capacity q_{∞} of the adsorbent for the enzyme. This has been confirmed [8].

The desorption reaction was performed under conditions of high ionic strength. It is known that at high salt concentrations (1 M sodium chloride) in a buffer with a pH of 6.0, desorption takes place [6]. This apppears to be also true in a buffer with a pH of 3.8.

The difference between the diffusion coefficients obtained in experiments with beads with non-adsorbed enzyme and in desorption experiments (1.5×10^{-7} cm^2/s and 1.8×10^{-7} cm^2/s respectively) is acceptable.

The desorption reaction is controlled by the diffusion of the enzyme in the beads. The diffusion coefficient was calculated as 1.8×10^{-7} cm^2/s. From the desorption experiments it was found that at concentrations of sodium chloride lower than 0.5 M the desorption reaction was retarded, probably because the reaction kinetics become an important factor in the desorption rate. Furthermore at lower sodium chloride concentrations desorption is not complete.

In a continuous process the alginate particles showed considerable potential for the isolation of the enzyme from an enzyme mixture. The loading capacity was constant during this experiment suggesting that no fouling or breakdown of the alginate occurred during the repeated process.

Furthermore the enzyme was purified up to a factor 3.0. The next step will be to apply this technique to a whole fermentation broth to study the isolation of the enzyme, since it is clear that isolation and purification of the enzyme from a fairly complex mixture is possible as a single process step.

Acknowledgement

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NOMENCLATURE

a	parameter defined by eqn. (6)
A	batch liquid fraction, defined by eqn. (5b) (-)
B	$q(t)/q_{\infty}$ (-)
C	batch liquid concentration at equilibrium (U/ml)
C(t)	batch liquid concentration at time t (U/ml)
C(0)	initial concentration of enzyme in liquid phase (U/ml)
$C_l(\infty)$	concentration of enzyme in liquid phase at the end of the desorption reaction (U/ml)
$C_p(0)$	concentration of enzyme in bead at t = 0 (U/ml)
d	diameter (cm)
D_{eff}	effective diffusion coefficient of enzyme in particle (cm^2/s)
G	parameter defined by eqn. (5a)
h	height (cm)
n	number of beads (-)
q	average loading of the bead at equilibrium (U/g)
q(t)	amount of enzyme adsorbed at time t (U/g)
q_{∞}	enzyme capacity of support (U/g)
R	radius of particle (cm)
r_b	radial position of the protein boundary ($0<r_b<R$) (cm)
Ra	dimensionless radial position of protein boundary (-)
t	time (s)
t_{∞}	time for enzyme to reach center of the bead at constant enzyme liquid concentration (s)
t_i	dimensionless reaction time (-)
V	volume (ml)
V_l	volume liquid phase (ml)
V_s	volume solid phase (ml)

w	parameter defined by eqn. (7) (-)
x_n	parameter defined by eqn. (9) (-)
α	parameter defined by eqn. (10) (-)
ρ_b	$(1 - A)\rho_p$, particle bulk density (g/ml)
ρ_p	particle density (g/ml (particle))

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CHAPTER 3

MODELLING THE ADSORPTION OF ENDO-POLYGALACTURONASE ON ALGINATE BEADS

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SUMMARY

The adsorption kinetics of endo-polygalacturonase on alginate beads is modelled with a pore diffusion model. The reaction velocity appears to be determined by the diffusion velocity of the enzyme into the matrix. Several parameters appear to have an influence on the adsorption rate. The diffusion coefficient for endo-polygalacturonase appears to be concentration dependent (between 0.8×10^{-11} m^2/s and 3.0×10^{-11} m^2/s), as found by model calculations. This is most likely caused by an increasing diffusion resistance as a result of the increased loading of the adsorbent at increased bulk liquid concentrations of the enzyme during the adsorption process.

pH and ionic strength have a distinct effect on the adsorption kinetics. With increasing pH (from pH 3.3 to pH 5.0) a decrease in reaction velocity and enzyme-adsorbent interaction is observed, resulting in a decreased value of the calculated diffusion coefficient. This effect can be explained by increased repulsive forces between the enzyme molecules and the matrix and between individual enzyme molecules.

The effect of ionic strength is concentration dependent. At sodium chloride concentrations above 400 mM no binding is observed between the enzyme and the adsorbent. The reaction kinetics, at increasing ionic strength, are described with decreasing values for the diffusion coefficient (from 1.5×10^{-11} m^2/s to 0.7×10^{-11} m^2/s).

The pore diffusion model predicts the adsorption process more accurately with experimentally determined reaction parameters than a previously used shrinking core model.

Submitted for publication.

3.1 INTRODUCTION

Adsorption of polysaccharide degrading enzymes on their substrates is a known method to isolate these enzymes from various sources [1]. Endo-polygalacturonase (endo-PG), pectate lyase, cellulase and α-amylase were for example successfully isolated using crosslinked pectate, cellulose and starch respectively as an adsorbent [2-3].

Adsorption of endo-polygalacturonase on alginate, crosslinked with epichlorohydrin, is also a method to isolate this enzyme from various sources [1,2,4,5]. The adsorption of the enzyme appeared to be influenced mainly by two parameters: the pH and the ionic strength of the adsorption medium [4,5]. Adsorption conditions are characterized typically by pH values between 3.3 and 5.0 and a low ionic strength (below 200 mM sodium chloride), whereas desorption can be accomplished at higher pH values (pH 6) and in higher ionic strength media (starting from 200 mM sodium chloride).

Alginate can be obtained in a bead form by preparing the Ca^{2+}-complex [6]. The use of alginate beads as the adsorbent in the isolation process allows the use of fluidized bed separations to isolate the enzyme from a medium [5]. In principle this method can be applied directly to complex mixtures, e.g. fermentation broths including the cell mass.

Although numerous models, taking the kinetics and diffusion phenomena into account, were developed for the adsorption of proteins on porous media [7-13], very few of these studies contain an experimental validation of the theoretical results. Recently a systematic description of the affinity adsorption of immunoglobulin G to protein A-agarose was presented [14].

The adsorption and diffusion process of endo-PG in alginate beads can be described with the commonly used shrinking core model [5,15,16]. This model is characterized by the assumption that the process is governed by the diffusion velocity of the enzyme into the matrix combined with a saturation type adsorption isotherm for the adsorbent-protein interaction. Furthermore the adsorption of the enzyme on the matrix is considered to be much faster than the diffusion and thus the kinetics of the adsorbate-adsorbent complex formation are neglected. From these assumptions it follows that the enzyme penetrates the particle as a front with a loading value equal to the saturation capacity. Further the loading pattern is characterized by an empty inner core that has not yet been reached by the enzyme.

The shrinking core model describes the adsorption process reasonably well but some deviations were found which were attributed to the fact that the adsorption isotherm is not of the high affinity type [5]. For systems where the

equilibrium isotherm does not resemble a saturation type isotherm other models may predict the adsorption process better [12,14]. One of these models is the pore diffusion-equilibrium adsorption model in which the adsorption isotherm can be of the Langmuir type and in which the adsorption rate is assumed to be governed by a kinetic term. The adsorption rate is dependent on the enzyme concentration in the pore liquid as well as on the concentration of the amount of enzyme bound to the adsorbent.

In this study the application of the pore diffusion-equilibrium adsorption and shrinking core models are studied for the endo-PG/alginate system. The models are used to describe the experimentally determined adsorption data and are compared for their ability to describe the adsorption reaction under different conditions with the experimentally determined parameters. In this way it should be possible to study influences such as pH, ionic strength and loading in a systematic way, resulting in the determination of the rate limiting step in the adsorption process under study as well as in a revelation of underlying phenomena.

3.2 THEORY

Mass transfer mechanisms

Generally four mechanisms can be distinguished considering the adsorption rate of proteins onto a porous matrix:

a. liquid film transport

The mass transfer between the bulk liquid and the surface of the adsorbent is given by eqn. 1:

$$F_{lf} = k_l (c_b - c_{l,p}) \qquad (1)$$

b. pore diffusion

The diffusion in the pores of the adsorbent is assumed to be described by Ficks law:

$$F_{pd} = -D_p \frac{dc}{dr} \qquad (2)$$

c. adsorption kinetics

When Langmuir type adsorption is assumed the adsorption rate is given by

eqn. 3:

$$v_i = k_i.c \left(1 - \frac{q}{q_m}\right) - k_d \left(\frac{q}{q_m}\right) \qquad (3)$$

d. surface diffusion

Surface diffusion is defined as the transport of the adsorbate in the adsorbed state (eqn. 4):

$$F_{sd} = -D_s \frac{dq}{dr} \qquad (4)$$

Adsorption isotherms

The adsorption isotherm equation is derived from eqn. 3 by substituting $v_i = dq/dt = 0$, yielding

$$q = q_m \frac{c}{K_s + c} \qquad (5)$$

The capacity of the support (q_m) and the Langmuir constant K_s (i.e. the enzyme concentration at which $q = \frac{1}{2}q_m$) can be calculated using this equation.

Adsorption models

a. Extended pore diffusion model

The model described here was derived for several adsorption systems [8-10]. The main features of this model are: (i) Both adsorption kinetics and diffusion mechanisms can be rate limiting in the adsorption process; (ii) the adsorption-desorption process is described by a kinetic term in which the binding velocity is dependent on the enzyme concentration in the matrix pores as well as on the amount already adsorbed on the support (eqn. 3); (iii) external mass transfer limitation in the liquid film is incorporated in the model.

The mass balances for the enzyme concentrations in the bulk liquid, adsorbed on the adsorbent and in the pore liquid are given by the following equations:

$$V_l \frac{dc_b(t)}{dt} = k_l \frac{3m_p}{\rho_p R} \left[\frac{c(R,t)}{P_e} - c_b(t)\right] \qquad (6)$$

with $c_b(0) = c_{b,0}$;

$$\frac{\delta q(r,t)}{\delta t} = v_i \tag{7}$$

with $q(r,0) = 0$;

$$\frac{\delta c(r,t)}{\delta t} = \frac{1}{r^2} \times \frac{\delta}{\delta r} \left[r^2 \times D_{eff,pd} \times \frac{\delta c(r,t)}{\delta r} \right] - v_i \tag{8a}$$

with

$$D_{eff,pd} \frac{\delta c(R,t)}{\delta r} = k_l \left[c_b(t) - \frac{c(R,t)}{P_e} \right] \tag{8b}$$

$$\frac{\delta c(0,t)}{\delta r} = 0; \quad c(r,0) = 0 \tag{8c}$$

The local adsorption velocity is given by eqn. 3. $D_{eff,pd}$ represents the effective pore diffusion coefficient, a parameter in which the contributions of the surface diffusion (D_s) and pore diffusion (D_p) are lumped together [17].

Changing to dimensionless variables:

$$C_b = \frac{c_b}{c_{b,0}}; \qquad C = \frac{c}{c_{b,0} \times P_e}; \qquad C_s = \frac{q}{q_m}; \tag{9a}$$

$$x = \frac{r}{R}; \qquad t_d = \frac{D_{eff,pd} \times t}{R^2}; \tag{9b}$$

$$\Phi^2 = \frac{R^2 \times k_i}{D_{eff,pd}}; \qquad \Theta = \frac{k_d}{k_j \times c_{b,0} \times P_e}; \qquad \Psi = \frac{c_{b,0} \times P_e}{q_m}; \tag{9c}$$

$$Bi = \frac{k_l \times R}{D_{eff,pd} \times P_e}; \quad \nu = \frac{m_p \times P_e}{V_l \times \rho_p}; \tag{9d}$$

Substitution of these variables and parameters in eqns. (6-8) yields the dimensionless model:

$$\frac{dC_b(t_d)}{dt_d} = -3\nu Bi\ [C_b(t_d) - C(1,t_d)] \tag{10a}$$

with $C_b(0) = 1$;

$$\frac{\delta C_s(x,t_d)}{\delta t_d} = \Psi\Phi^2 \{C(x,t_d)[1 - C_s(x,t_d)] - \Theta C_s(x,t_d)) \tag{10b}$$

with $C_s\ (x,0) = 0$;

$$\frac{\delta C(x,t_d)}{\delta t_d} = \frac{1}{x^2}\frac{\delta}{\delta x}\left[x^2 \frac{\delta C(x,t_d)}{\delta x}\right] - \frac{1}{\Psi}\frac{\delta C_s(x,t_d)}{\delta t_d} \tag{11a}$$

with

$$\frac{\delta C(1,t_d)}{\delta x} = \frac{1}{3\nu}\frac{dC_b(t_d)}{dt_d} \tag{11b}$$

$$\frac{\delta C(0,t_d)}{\delta x} = 0; \quad C(x,0) = 0. \tag{11c}$$

This set of equations (10-11) can be solved assuming a quasi-steady-state approximation for diffusion in the particle, i.e. $\delta C(x,t_d)/\delta t_d = 0$. This is valid for $\nu << 1$. In this case the model can be rearranged and solved according to Do and Bailey [8,18,19] by numerical integration for which purpose a computer program was developed.

b. Shrinking core model

As a limiting case for the model described above the shrinking core model is derived. Using this model the following assumptions are made (i) the rate of adsorption is much higher than the diffusion velocity (i.e. the adsorption is at

equilibrium and the system is fully diffusion controlled); (ii) the diffusion coefficient ($D_{eff,sc}$) is constant during the experiment; (iii) the protein adsorption capacity of the support is much larger than the amount of protein dissolved in the pores of the support; (iv) the protein is irreversibly adsorbed to the support and (v) the isotherm governing the loading process is of the saturation type (i.e. for $c>0$, $q=q_m$ and for $q<q_m$, $c=0$).

When spherical beads (radius R) are suspended in a finite solution with an initial enzyme concentration C(0) the bulk liquid concentration is given by [15,16]:

$$\frac{D_{eff,sc} \times (1-A) \times t}{R^2 \times A} = -\frac{1}{3}\ln\left(\frac{a^3 + w^3}{a^3 + 1}\right) + \frac{1}{6a}\ln\left(\frac{(a^2 - a + 1)(a + w)^2}{(a^2 - aw + w^2)(a + 1)^2}\right) + \frac{1}{a \times 3^{1/2}}\tan^{-1}\left(\frac{2 - a}{a \times 3^{1/2}}\right) - \tan^{-1}\left(\frac{2w - a}{a \times 3^{1/2}}\right) \quad (12)$$

where

$$A = \frac{V_l}{V_l + (m_p/\rho_p)} \quad (13)$$

$$G = \frac{A \times C(0)}{q_m} \quad (14)$$

and

$$a = (G-1)^{1/3} \qquad G > 1 \quad (15a)$$

$$a = 0 \qquad G = 1 \quad (15b)$$

$$a = -(1-G)^{1/3} \qquad G < 1 \quad (15c)$$

and

$$w = [1-G\{1-C(t)/C(0)\}]^{1/3} \quad (16a)$$

$$= [1-q(t)/q_m]^{1/3} \quad (16b)$$

Film transport and adsorption kinetics are neglected in this model which can be a valid assumption as will be discussed later.

Determination of the pore diffusion coefficient

The pore diffusion coefficient was determined experimentally, ($D_{eff,exp}$), as described before [5] by fitting the experimental results with the equation for diffusion of substrates out of a sphere:

$$C(t) = \frac{C_p(0)}{(1+\alpha)}\left\{1 - \sum_{n=1}^{\infty} \frac{6\alpha(1+\alpha)\exp(-D_{eff,exp}x_n^2 t/R^2)}{9 + 9\alpha + x_n^2\alpha^2}\right\} \quad (17a)$$

or

$$\frac{C(t)}{C_l(\infty)} = 1 - \sum_{n=1}^{\infty} \frac{6\alpha(1+\alpha)\exp(-D_{eff,exp}x_n^2 t/R^2)}{9 + 9\alpha + x_n^2\alpha^2} \quad (17b)$$

x_n represents the non-zero positive roots of:

$$\tan x_n = \frac{3\,x_n}{3 + \alpha x_n^2} \quad (18)$$

$$\alpha = \frac{V_l}{n \times 4\pi R^3/3} \quad (19)$$

$C_l(\infty)$ is related to $C_p(0)$ as:

$$C_l(\infty) = \frac{C_p(0)}{(1+\alpha)} \quad (20)$$

Under these non binding conditions, where surface diffusion is not of importance, the value of $D_{eff,exp}$ should represent the value of the pore diffusion coefficient D_p.

3.3 MATERIALS AND METHODS

Materials

Sodium alginate (Manucol DM) was obtained from Alginate Industries (London, UK). Rapidase, a pectolytic enzyme preparation was obtained from

Gist-brocades (Seclin, France).

Bead preparation

Alginate powder is solubilized by using an ultraturrax mixer to a 2% (w/v) alginate solution in distilled water. To prepare beads the solution is passed through a dropping funnel into a continuously stirred 200 mM calcium chloride solution. Beads with different diameters are obtained by varying the opening of the needle. After 24 h of complexation the beads are removed from the solution and stored in a 100 mM sodium acetate buffer of pH 3.8 with 10 mM calcium chloride. Bead diameters are determined as described previously [5].

Adsorption experiments

a. isotherms

Adsorption isotherms are determined by incubating 0.5 g of beads with varying amounts of enzyme (17 - 510 U/ml). The adsorption time is 48 h. The amount of adsorbed enzyme is calculated from the difference between the enzyme concentration at t = 0 and at the end of the experiment. In all media 10 mM calcium chloride is present.

b. kinetics

Adsorption kinetics are determined at room temperature in a 100 mM sodium acetate buffer pH 3.8 with 10 mM calcium chloride. In the measurements several parameters are varied to obtain results used in the model calculations. Typically the experiments are performed in 100 ml of adsorption buffer. The amount of alginate, the radius of the particles and the initial enzyme concentration in the liquid are varied. The adsorption kinetics were followed by taking liquid samples. Enzyme activity analysis was performed as described earlier [5].

Additional experiments were performed with 100 mM sodium acetate at pH 3.3, 4.2, 4.6 and 5.0 respectively and with 100 mM sodium acetate at pH 3.8 with 25 mM, 50 mM, 100 mM and 200mM sodium chloride respectively.

3.4 RESULTS AND DISCUSSION

Adsorption isotherms

Adsorption isotherms were determined for various reaction conditions.

The adsorption capacity of endo-PG on alginate beads is dependent on ionic

strength and pH (Fig. 3.1). Table 3.1 contains the values for q_m and K_S for isotherms determined at different pH and ionic strength values. The values of K_S show that the isotherms are not of the "high affinity type" in the low to medium bulk concentration region (up to 500 × 10^6 U/m^3. The effect of pH and ionic strength on the adsorption equilibrium is twofold. An increase in pH

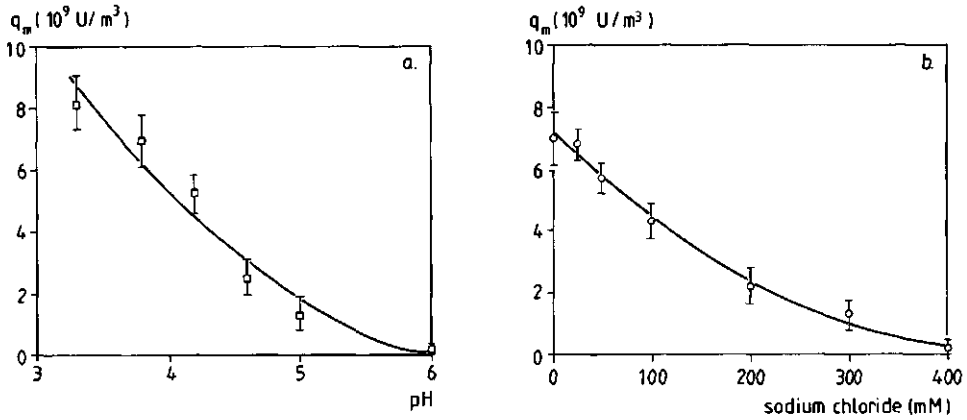

Fig. 3.1 - Dependance of the capacity of the adsorbent (q_m) on the pH and the ionic strength.

Table 3.1 - Adsorption isotherms of endo-PG/alginate system.

pH	NaCl (mM)	q_m (10^9 U/m^3)	K_S/q_m	K_S (10^6 U/m^3)
3.8	0	7.0 ± 0.9	0.007 ± 0.002	45±15
3.8	25	6.8 ± 0.5	0.007 ± 0.001	48±8
	50	5.7 ± 0.5	0.012 ± 0.002	70±10
	100	4.3 ± 0.6	0.026 ± 0.006	110±30
	200	2.2 ± 0.6	0.116 ± 0.009	260±70
	300	1.2 ± 0.5	0.45 ± 0.1	550±300
	400	0.3 ± 0.2	1.0 ± 0.2	300±200
3.3	0	8.2 ± 0.8	0.007 ± 0.002	60±20
4.2	0	5.3 ± 0.6	0.019 ± 0.005	100±20
4.6	0	2.5 ± 0.5	0.066 ± 0.004	170±40
5.0	0	1.3 ± 0.4	0.31 ± 0.06	400±150
6.0	0	0.2 ± 0.1	2.0 ± 0.4	400±200

(going up from pH 3.3) as well as an increase in ionic strength yield a decrease in adsorption capacity and strength of interaction, reflected in decreasing values for q_m and increasing values for K_S, respectively. At an ionic strength above 400 mM sodium chloride in 100 mM sodium acetate pH 3.8 the adsorption reaches the zero level. Most of the experiments discussed in this study are performed in 100 mM sodium acetate pH 3.8 with 10 mM calcium chloride. From the comparable ionic strength data in Table 3.1 and literature [4,5] it is clear that these conditions lead to good adsorption. Addition of calcium chloride is necessary to stabilize the beads. Calcium chloride in itself has the same influence on the adsorption parameters as sodium chloride [4].

Film transport coefficient (k_f) and adsorption rate constant (k_i)

Although the overall adsorption rate of proteins in porous media is governed most frequently by diffusion, in the initial phase of the process external mass transport and/or the rate of the protein/matrix complex formation can be of importance. For both cases methods were derived to estimate either k_f [20] or k_i [19] from the initial adsorption rate. For the case where k_f is rate limiting Do derived [20]:

$$1 - \frac{c_b(t)}{c_{b,0}} = \frac{3m_p}{\rho_p \times V_l}\left(\frac{k_f \times t}{R}\right) \qquad (21)$$

with the following criterion:

$$\frac{V_l(c_{b,0} - c_b(t))}{3m_p \times q_m} << 1 \qquad (22)$$

The value of k_f was obtained from experiments meeting these demands (Fig. 3.2) yielding for k_f values of 1.2×10^{-6} m/s and 1.3×10^{-6} m/s respectively. This value corresponds very well with literature data for proteins [14,20].

Kinetics of complex formation will only be rate determining when

$$k_i \times R << k_f \qquad (23)$$

For cases where k_i is considered to be rate determining in the initial phase of the adsorption reaction, Do and Bailey [19] derived a correlation using a plot of $-\log (c_b/c_{b,0})$ versus the dimensionless time τ by relating the

initial slope of the curve with the Thiele-modulus (Φ), based on the effective diffusion coefficient (D_{eff}), with

$$\tau = \frac{m_p \times P_e \times D_{eff}}{V_l \times \rho_p \times R^2} \times t \qquad (24)$$

and

$$\Phi = (R^2 k_i / D_{eff})^{1/2} \qquad (25)$$

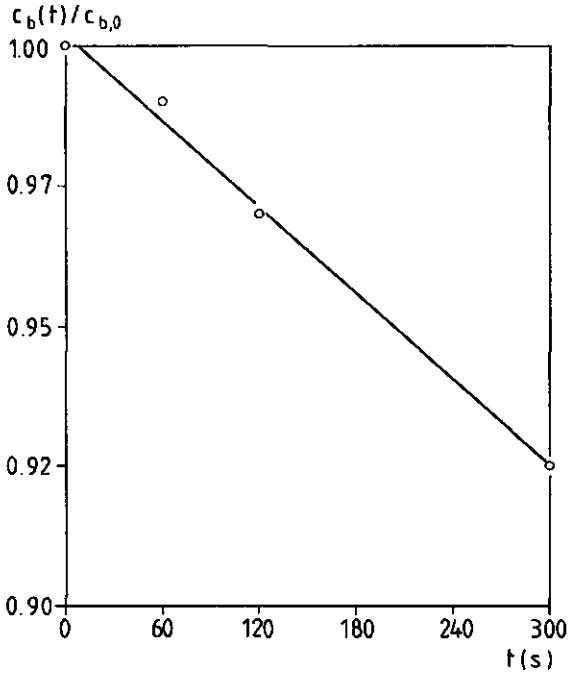

Fig. 3.2 - Determination of k_l for the adsorption of endo-PG on alginate beads. V_l: 100 × 10^{-6} m^3; $c_{b,0}$ = 100 × 10^6 U/m^3; m_p = 5 × 10^{-3} kg.

When this method was used for analyzing the experimental data a value of 2.4/s for k_i was calculated. Using eqn. 23 this shows that external mass transfer is rate determining in the initial phase of the reaction.

Diffusion coefficient

The effective pore diffusion coefficient was determined under non binding conditions (pH 6.0) with fully equilibrated beads as described previously [5]. Surface diffusion can be expected to be negligible under these conditions. For

beads with varying concentrations of alginate (0.5 - 3.0 % (w/v)) the diffusion coefficient varies between 4.5×10^{-11} and 1.0×10^{-11} m^2/s. In this study 2 % (v/v) alginate beads were used, the experimentally determined effective pore diffusion coefficient in this case being 1.5×10^{-11} m^2/s (Fig. 3.3).

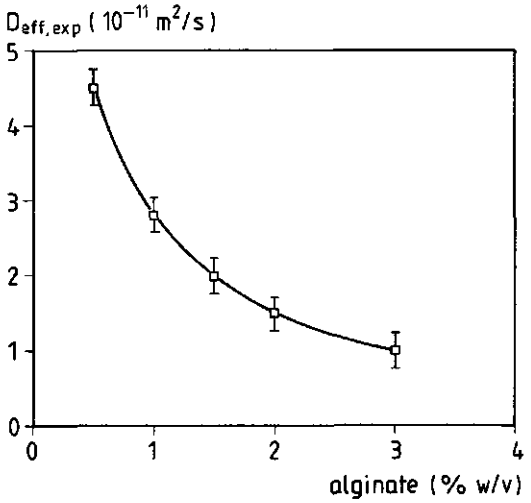

Fig. 3.3 - Diffusion coefficient ($D_{eff,exp}$) of endo-PG in alginate beads as a function of alginate concentration. Conditions: see reference [5].

Determination of the rate limiting step at pH 3.8

Dimensionless variables were calculated to characterise the adsorption process and the adsorption rate determining step. Experimentally determined data together with literature data were used. In Table 3.2 the data for the endo-PG/alginate system are collected. Relevant information with regard to the rate limiting step is obtained using the dimensionless parameters given in this table. The Thiele modulus (Φ) (i.e. the ratio between rate of binding and the diffusion rate (see eqn. 9c)) and the value of Bi (the ratio between the film transport and pore diffusion (see eqn. 9d)) indicate that the film transport and the rate of binding will not determine the overall rate of adsorption at pH 3.8. However, in the initial stage of the adsorption process the overall adsorption rate will be determined by the liquid film transport and the binding kinetics of the enzyme/matrix complexation as shown in the preceding paragraph [12-14].

Table 3.2 - Reaction parameters for endo-PG/alginate system at pH 3.8.

adsorbent	**value**
ρ_p	: 1.006×10^3 kg/m^3
q_m	: 7.0×10^9 U/m^3
R	: 0.75×10^{-3} m
equilibrium and rate parameters	
k_l	: 10^{-6} - 10^{-5} m/s[a]
D_p ($D_{eff,exp}$)	: 1.5×10^{-11} m^2/s
D_s	: 10^{-13} - 10^{-12} m^2/s[b]
K_s	: 45×10^6 U/m^3
k_i	: $\geq$ 0.0018 1/s
k_d	: $\leq 8.1 \times 10^4$ U/m^3.s[c]
dimensionless parameters[d]	
Φ^2	: $\geq$ 67
Bi	: 50 - 500
λ	: 0.93 - 9.3[e]
	: 0.19 - 1.9[f]

[a] : derived from ref. [20]; [b] : taken from ref. [21]; [c] : calculated with values of K_s and k_i; [d] : calculated with parameters from table and $C_{b,o} = 50 \times 10^6$ U/m^3; [e] : $C_{b,0} = 50 \times 10^6$ U/m^3; [f] : $C_{b,0} = 250 \times 10^6$ U/m^3.

The relative importance of surface to pore diffusion [12] is characterized by

$$\lambda = \frac{D_s \times q_m}{D_p \times c_{b,o}} \qquad (26)$$

Numerical data with respect to surface diffusion are scarce. The value of λ (Table 3.2), calculated with the value for D_s for surface diffusion of albumin on glass surfaces [21], indicates that pore diffusion as well as surface diffusion may be of importance during the adsorption reaction. Especially at low adsorbate concentrations (50×10^6 U/m^3) surface diffusion may be an important parameter in the adsorption process.

Modelling of the adsorption reaction

The experimental adsorption reaction data were fitted to obtain the diffusion coefficients using the previously described models. The concentration of the adsorbent (m_p), the radius of the adsorbent (R) and the initial concentration of enzyme ($c_{b,0}$) respectively, were varied in the experiments. The rate parameter k_i was set on 5/s, for q_m and K_S the experimentally determined values (7.0×10^9 U/m^3 and 45×10^6 U/m^3) were taken. So the reaction kinetics of the complex formation is assumed to be not rate limiting. Table 3.3 gives the results obtained for the various reaction conditions.

Variation of alginate concentration

Variation of the amount of alginate beads results in a fairly constant value for the calculated effective diffusion coefficient in the extended pore diffusion model (Table 3.3A). This value is comparable to $D_{eff,exp}$ (see also "Variation of initial enzyme concentration"). Fig. 3.4(a) gives the model fits for the extended pore model. The shrinking core model gives values for the $D_{eff,sc}$

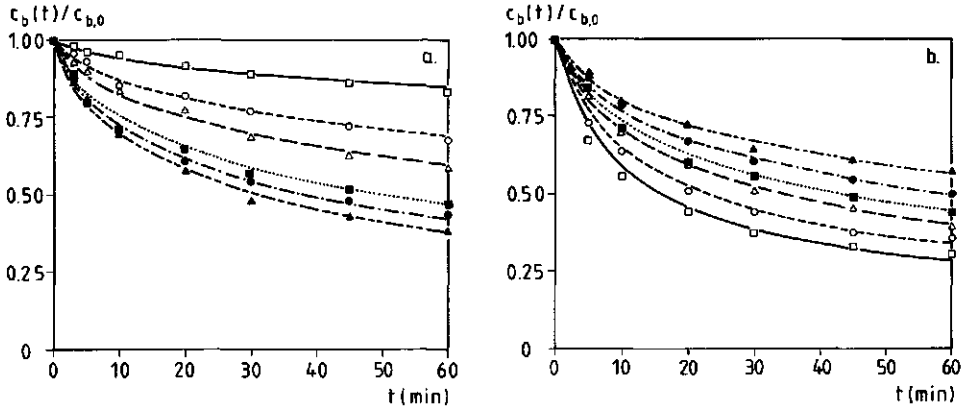

Fig. 3.4 - Model fits for the adsorption of endo-PG on alginate beads with the extended pore diffusion model. (a) Variation of amount of alginate (m_p/V_l). □ : 50 kg/m^3; ○ : 100 kg/m^3; △ : 150 kg/m^3; ■ : 200 kg/m^3; ● : 250 kg/m^3; ▲ : 300 kg/m^3; $c_{b,0} = 400 \times 10^6$ U/m^3. Other parameters as given in Tables 3.1, 3.2 and 3.3.
(b) Variation of initial enzyme concentration. □ : $c_{b,0} = 40 \times 10^6$ U/m^3; ○ : $c_{b,0} = 80 \times 10^6$ U/m^3; △ : $c_{b,0} = 160 \times 10^6$ U/m^3; ■ : $c_{b,0} = 230 \times 10^6$ U/m^3; ● : $c_{b,0} = 310 \times 10^6$ U/m^3; ▲ : $c_{b,0} = 395 \times 10^6$ U/m^3; R = 0.69×10^{-3} m. Other parameters as given in Tables 3.1, 3.2 and 3.3.

Table 3.3 - Extended pore diffusion model and shrinking core model: calculated diffusion coefficient at various reaction conditions[a].

$c_{b,0}$ (10^6 U/m^3)	m_p/V_l (kg/m^3)	R_{bead} (10^{-3} m)	$D_{eff,pd}$ (10^{-11} m^2/s)	$D_{eff,sc}$
A. variation of alginate concentration				
140	100	0.72±0.01	1.4±0.3	0.52±0.1
140	150	0.72±0.01	1.3±0.3	0.37±0.1
140	200	0.72±0.01	1.1±0.2	0.26±0.1
400	50	0.69±0.03	1.0±0.2	0.56±0.1
400	100	0.69±0.03	1.1±0.2	0.60±0.1
400	150	0.69±0.03	0.9±0.2	0.50±0.1
400	200	0.69±0.03	1.1±0.2	0.48±0.1
400	250	0.69±0.03	0.9±0.2	0.36±0.1
400	300	0.69±0.03	0.8±0.2	0.32±0.1
B. variation of particle radius				
100	150	1.11±0.03	2.3±0.5	0.55±0.1
100	150	0.93±0.03	1.7±0.4	0.41±0.1
100	150	0.83±0.04	1.6±0.4	0.40±0.1
100	150	0.69±0.03	1.7±0.4	0.33±0.1
315	200	1.87±0.05	0.9±0.2	0.38±0.1
335	200	1.57±0.05	0.9±0.2	0.36±0.1
365	200	1.01±0.04	0.8±0.2	0.36±0.1
390	200	0.88±0.04	1.0±0.2	0.50±0.1
390	200	0.80±0.03	0.9±0.2	0.42±0.1
390	200	0.69±0.03	0.8±0.2	0.38±0.1
C. variation of initial enzyme concentration				
25	150	0.72±0.01	2.8±0.6	
40	150	0.69±0.03	2.2±0.5	
50	150	0.72±0.01	2.2±0.5	
80	150	0.69±0.03	1.7±0.3	
95	150	0.72±0.01	1.8±0.4	
160	150	0.69±0.03	1.5±0.3	
190	150	0.72±0.01	1.2±0.3	
230	150	0.69±0.03	1.3±0.3	
310	150	0.69±0.03	1.3±0.2	
395	150	0.69±0.03	1.0±0.2	

[a]: Experimental conditions: Buffer: 100 mM sodium acetate pH 3.8 with 10 mM calciumchloride at room temperature.

which are three to five times lower than the value for $D_{eff,exp}$. Furthermore an undesirable decrease in the diffusion coefficient with increasing alginate concentration is observed with the shrinking core model. This can be explained by the fact that this model assumes a saturation type adsorption isotherm

which means that the loading of the particle is going on with a sharp front with the q_m given by the isotherm. From Table 3.1 (K_s) it is clear that the adsorption isotherm does not resemble a saturation type isotherm in the bulk liquid enzyme concentrations under study. From these results it can be seen that the extended pore diffusion model is more consistent and accurate than the shrinking core model in predicting the adsorption reaction.

Variation of bead radius

When the bead radius is varied the adsorption process can be described very well with the experimentally determined reaction parameters (see Table 3.3B). The calculated values of $D_{eff,pd}$ are around 1.0 - 1.5 $\times$ 10^{-11} m^2/s for most cases indicating that pore diffusion is the rate determining transport mechanism. The apparent dependency of the calculated value of $D_{eff,pd}$ on the

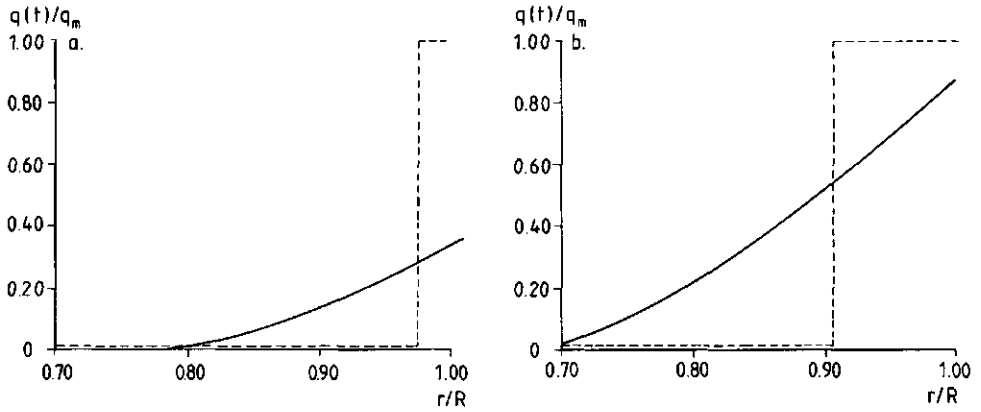

Fig. 3.5 - Calculated adsorption profiles of endo-PG on alginate beads according to the shrinking core model and the pore diffusion model. (a) m_p = 15 $\times$ 10^{-3} kg; V_l = 100 $\times$ 10^{-6} m^3; R = 0.69 $\times$ 10^{-3} m; (b) m_p = 5 $\times$ 10^{-3} kg; V_l = 100 $\times$ 10^{-6} m^3; R = 0.69 $\times$ 10^{-3} m; Shrinking core model (t = 120 min):- - - - - - - -; Pore diffusion model (t = 120 min.):———— ; q_m = 7000 $\times$ 10^6 U/m^3; $c_{b,0}$ = 400 $\times$ 10^6 U/m^3.

bulk liquid concentration at the start of the experiment ($c_{b,0}$) will be discussed in the next paragraph. Again as observed and explained in the former paragraph the calculated values for $D_{eff,sc}$, being constant in the whole particle diameter region, are lower than the calculated values in the extended

pore diffusion model. Fig. 3.5 shows the calculated adsorption profiles in the adsorbent for the two models after 120 min (R = 0.69 × 10^{-3} m).

Variation of initial enzyme concentration

When the initial enzyme concentration is changed the calculated values for $D_{eff,pd}$ and $D_{eff,sc}$ vary (Table 3.3C). The model fits of the pore diffusion model are given in Fig. 3.4(b). With decreasing initial enzyme concentration the calculated effective diffusion coefficient increases indicating that surface diffusion may be an important parameter in the process.

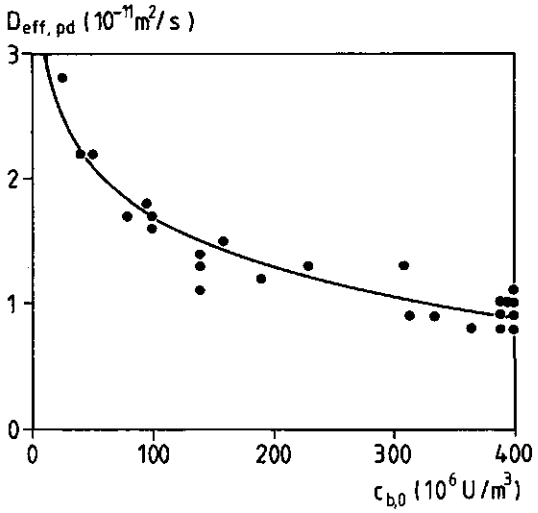

Fig. 3.6 - $D_{eff,pd}$ as a function of $c_{b,0}$.

Surface diffusion is expected to be more predominant at the low concentrations [12,17,22]. This is confirmed by the results; the calculated diffusion coefficient is larger than the value measured for free diffusion (1.5 × 10^{-11} m^2/s) of the enzyme in the beads (Table 3.3 and Fig. 3.3). However, at higher values for $c_{b,0}$ the decrease of the calculated diffusion coefficient continues to values lower than the experimentally found value for D_{eff} (see Section "Diffusion coefficient"). The calculated value of $D_{eff,pd}$ as a function of $c_{b,0}$ slowly levels of to values of approximately 0.9 × 10^{-11} m^2/s at $c_{b,0}$ = 400 × 10^6 U/m^3 (Fig. 3.6). The difference between $D_{eff,pd}$ and $D_{eff,exp}$ might be due to experimental accuracy, however it is more likely that another

mechanism is involved in the adsorption reaction. Earlier results [5] showed that at constant enzyme bulk liquid concentration adsorption is slowed down during the experiment, to be explained by hindered diffusion at increased enzyme concentrations on the adsorbent. The decreasing value of $D_{eff,pd}$ can also be due to this mechanism. Hindered diffusion due to loading of the outer part of the bead then results in lower values of $D_{eff,pd}$. According to this mechanism, being absent at desorption conditions, the value of $D_{eff,exp}$ determined under non binding conditions should be independent of the concentration. Desorption profiles were determined over a large concentration range (50 - 1000 $\times$ 10^6 U/m^3) and the value of $D_{eff,exp}$ was indeed measured to be constant. So the observed changes might rather be due to additional diffusion resistance of the protein/matrix complex.

Changes in the diffusion behaviour of proteins into and from alginate beads are reported in literature [23]. The diffusion of albumin, γ-globulin and fibrinogen into and from alginate beads, probably as a result of the experimental procedure, were found to be different. When the proteins were included in the beads during the preparation process , diffusion from 2% (w/v) alginate beads occurred for all three proteins. However, the diffusion of the substrates into the "empty" 2% (w/v) gel beads was restricted or did not occur at all. It was suggested that due to the inclusion of protein in an alginate matrix during complex formation the alginate complex gets more porous than an alginate complex without addition of proteins. We did determine the value of $D_{eff,exp}$ likewise, i.e. the parameter is determined from experiments in which the enzyme was included in the matrix during bead preparation [5]. This might be another explanation for the difference in the diffusion coefficients.

Variation of pH and ionic strength

pH and ionic strength are the most important parameters determining the equilibrium and reaction kinetics of the system [4,5,24]. The experimental results at pH 3.3 and 3.8 and at sodium chloride concentrations up to 50 mM are quite well described with the experimentally determined loading capacity obtained from the isotherm when assuming diffusion to be the rate determining parameter (Table 3.4).

Going up from pH 4.2 the calculated diffusion coefficient decreases strongly and therewith deviates from the experimentally determined value of 1.5 $\times$ 10^{-11} m^2/s for $D_{eff,exp}$. A concentration dependency, as explained earlier, of the diffusion coefficient is present for all reaction conditions except at pH 5.0 where no significant difference was found.

Table 3.4 - Adsorption of endo-PG on alginate beads as a function of pH and ionic strength: calculated diffusion coefficients in the extended pore model[a].

pH	$c_{b,0}$ (10^6 U/m^3)	sodium chloride (mM)	q_m[b] (10^6 U/m^3)	$D_{eff,pd}$ (10^{-11} m^2/s)
3.3	50	-	8200	3.0±0.6
3.8	50	-	7000	2.5±0.5
	200	-	7000	1.4±0.3
4.2	50	-	5300	2.2±0.3
	200	-	5300	0.9±0.2
4.6	50	-	2500	0.9±0.2
	200	-	2500	0.3±0.1
5.0	50	-	1300	0.5±0.2
	200	-	1300	0.5±0.2
3.8	50	25	6800	3.4±0.5
	200		6800	1.4±0.2
	50	50	5700	3.3±0.5
	200		5700	1.7±0.3
	50	100	4300	1.5±0.3
	200		4300	1.1±0.2
	50	200	2200	0.8±0.1
	200		2200	0.5±0.1

[a] R = (0.81±0.03) × 10^{-3} m; V_l: 100 × 10^{-6} m^3; amount of alginate: 15 × 10^{-3} kg; k_i: 5/s; Buffer: 100 mM sodium acetate with 10 mM calcium chloride; [b]: values obtained from adsorption isotherms.

One way to explain these results might be that the rate of reaction is of importance on the overall rate adsorption at increased pH values and ionic strength. The values of k_i and k_d for the different reaction conditions in which the adsorption kinetics seem to be important were estimated (Table 3.5)

by the best fit of the model on the experimental results using the value of K_S from Table 3.1. The diffusion coefficient is assumed to be concentration dependent being 2.5×10^{-11} m^2/s and 1.4×10^{-11} m^2/s for $C_{b,o} = 50 \times 10^6$ U/m^3 and $C_{b,o} = 200 \times 10^6$ U/m^3 respectively. The latter are the values found at pH 3.8 where diffusion is the rate determining mechanism (Table 3.3).

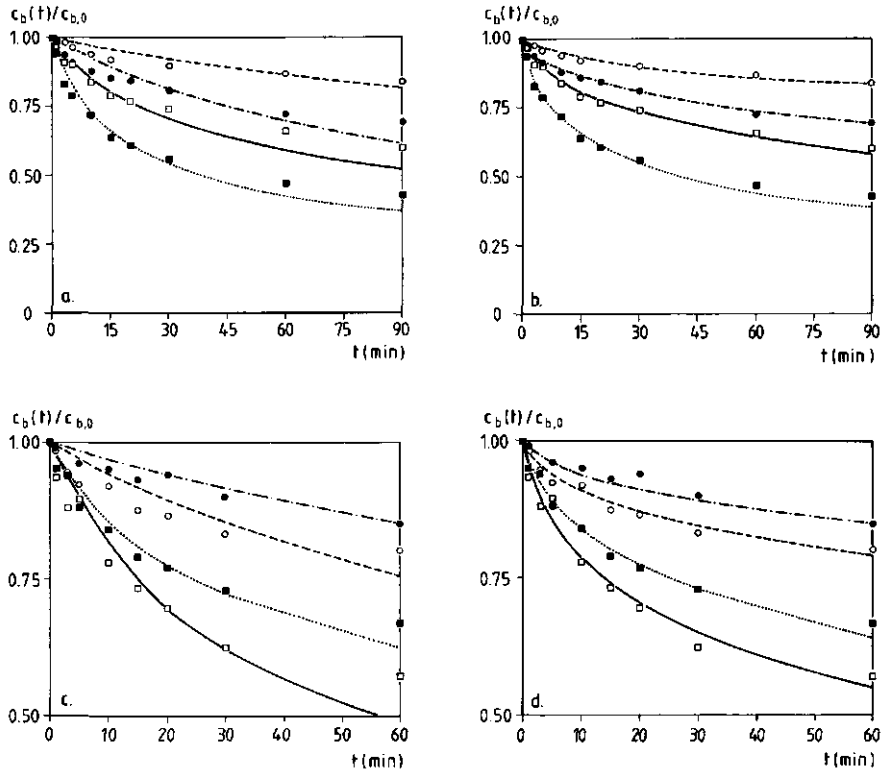

Fig. 3.7 - Model fits for the adsorption of endo-PG on alginate beads with the extended pore diffusion model. Influence of pH: (a) Fits with values from Table 3.5; (b) Fits with the parameters from Table 3.4. Results (experimental, model): ■ , · · · · · *: pH 4.2 ($c_{b,0}$ = 50 × 10^6 U/m^3);* □ , ——— *: pH 4.2 ($c_{b,0}$ = 200 × 10^6 U/m^3);* ● , •—•—: *pH 4.6 ($c_{b,0}$ = 50 × 10^6 U/m^3);* ○ , – – – –: *pH 4.6 ($c_{b,0}$ = 200 × 10^6 U/m^3). Influence of ionic strength: (c) Fits with values from Table 3.5; (d) Fits with the parameters from Table 3.4. Results (experimental, model):* □ , ——— *: 100mM ($c_{b,0}$ = 50 × 10^6 U/m^3);* ■ , · · · · · · *: 100 mM ($c_{b,0}$ = 200 × 10^6 U/m^3);* ○ , – – – –: *200 mM ($c_{b,0}$ = 50 × 10^6 U/m^3);* ● , •—•—•—: *200 mM ($c_{b,0}$ = 200 × 10^6 U/m^3).*

The quality of the fits is poor (Figs. 3.7 (a)/(c)) showing that the adsorption can not be described by assuming the rate of reaction to be governing the process. The fit is dominated in the first fifteen minutes by the

calculated rate constants k_i and k_d whereas after 60 min the diffusion coefficient is again predominant in the reaction and it then determines the adsorption level. This results in a too slow rate prediction in the first time span followed by overestimation of the adsorption in the second period of the reaction.

Table 3.5 - Calculated rate constants for the adsorption of endo-PG on alginate beads for the model fits from fig 7(a)/(c).

pH	sodium chloride (mM)	$c_{b,0}$ (10^6 U/m^3)	k_i (1/s)	k_d (10^7 U/m^3.s)	Φ (-)
4.2	0	50	1.4×10^{-1}	1.4	60.6
	0	200	4×10^{-2}	0.4	43.2
4.6	0	50	3×10^{-3}	0.05	8.8
	0	200	7×10^{-4}	0.012	5.7
5.0	0	50	5×10^{-4}	0.02	3.6
	0	200	7×10^{-4}	0.028	5.7
3.8	100	50	3×10^{-2}	0.33	28.1
		200	5×10^{-2}	0.55	48.4
	200	50	2×10^{-3}	0.052	7.2
		200	1×10^{-3}	0.026	6.8

D_{eff}: 2.5×10^{-11} m^2/s ($c_{b,0} = 50 \times 10^6$ U/m^3); 1.4×10^{-11} m^2/s ($c_{b,0} = 200 \times 10^6$ U/m^3).

The fits with the diffusion coefficient as the fitting parameter (Figs. 3.7 (b)/(d)) predict the adsorption kinetics much better. Yet a systematic underestimation in the first part (to be explained by k_i) but also an overestimation in the second part occurs. The last phenomenon indicates that the diffusion coefficient should decrease at increasing time, a phenomenon not found at pH 3.8 (Fig. 3.4). This means that a change in diffusion behaviour is more likely to be the explanation for the changing adsorption behaviour of the endo-PG on the alginate at increased pH, with calculated values for the

diffusion coefficient that are smaller than the ones determined experimentally at pH 3.8.

There are reasons why the diffusion can be influenced by pH. The endo-PG used has an isoelectric point of 3.2-3.5 [25] resulting in an increase of negative charge with an increase of pH. This may cause increasing repulsive effects between the matrix and the enzyme at increased pH thus hindering the diffusion of the enzyme into the beads [26]. As stated earlier the adsorbed enzyme itself also hinders the diffusion into the particle. With increasing pH this effect also will become more important due to stronger repulsion forces between the enzyme molecules.

This theory cannot explain the decrease in $D_{eff,pd}$ with increasing ionic strength. In fact electrostatic repulsion should decrease at increasing ionic strength resulting in improved diffusivity of the enzyme into the matrix. Another, yet unknown factor, affects the apparent diffusion of the enzyme in the matrix in this case.

3.5 CONCLUSIONS

The adsorption reaction of endo-PG on alginate beads is described. The pore diffusion model describes the adsorption reaction reasonably well. At all conditions the diffusivity of the enzyme into the matrix is the rate determining parameter. The calculated values for the diffusion coefficient are smaller than the value for this parameter in water [23,27]. The calculated diffusion coefficient is concentration dependent which is probably due to two mechanisms. At low enzyme bulk concentrations pore diffusion is accompanied by surface diffusion yielding values for D_{eff} which are larger than expected for pore diffusion only. At increased $c_{b,0}$ the diffusion may be obstructed due to fouling or steric hindrance resulting in a reduced diffusion velocity. It is shown that the diffusion coefficient of enzyme into the beads is somewhat smaller than that found for the diffusion of the enzyme from the beads, particularly when the enzyme was included during the alginate complex formation [23].

The diffusion coefficient appears to be also dependent on the physical conditions of the process medium (pH and ionic strength). Increase of pH and ionic strength results in a decrease of the diffusion coefficient.

The previously used shrinking core model is less accurate in predicting the adsorption characteristics. The diffusion coefficient is underestimated in all cases. Furthermore the variation in calculated diffusion coefficients is larger in

experiments with varying adsorbent concentrations. This can be explained by the fact that the loading of the adsorbent is assumed to be governed by the capacity value (q_m) of the matrix only, as it does not include the influence of the kinetics of complex formation.

NOMENCLATURE

a	: parameter defined in eqn. 15 (-)
A	: parameter defined in eqn. 13 (-)
Bi	: Biot number defined in eqn. 9d (-)
c	: local concentration of enzyme in the pore liquid phase (U/m^3)
c_b	: bulk liquid concentration (U/m^3)
$c_{b,0}$	: liquid concentration at t = 0 (U/m^3)
$c_{l,p}$	: concentration at liquid-particle interface (U/m^3)
C	: dimensionless local concentration of enzyme in the pore liquid phase (-)
C_b	: dimensionless bulk liquid concentration (-)
C_s	: dimensionless concentration of the enzyme on the adsorbent (-)
C(t)	: batch liquid concentration at time t (U/m^3)
$C_l(\infty)$	: concentration of enzyme in liquid phase at the end of the desorption reaction (U/m^3)
$C_p(0)$	: concentration of enzyme in bead at t = 0 (U/m^3)
D_{eff}	: effective pore diffusion coefficient of protein in particle (m^2/s)
$D_{eff,exp}$	: effective pore diffusion coefficient of protein in particle as derived from eqn. 17 (m^2/s)
$D_{eff,pd}$	: effective pore diffusion coefficient of protein in particle as derived from the extended pore diffusion model (m^2/s)
$D_{eff,sc}$	: effective pore diffusion coefficient of protein in particle as derived from the shrinking core model (m^2/s)
D_p	: diffusion coefficient of protein in particle pore (m^2/s)
D_s	: diffusion coefficient of protein due to surface diffusion (m^2/s)
D_w	: diffusion coefficient of protein in liquid phase (m^2/s)
F_{lf}	: flux from bulk liquid to liquid/particle interface ($U/m^2.s$)
F_{pd}	: flux through particle pores ($U/m^2.s$)
F_{sd}	: flux due to surface diffusion ($U/m^2.s$)
G	: parameter defined in eqn. 14 (-)
k_i	: rate constant local adsorption (1/s)
k_d	: rate constant local desorption ($U/m^3.s$)
k_l	: liquid film transport coefficient (m/s)

K_s : Langmuir constant (U/m^3)

m_p : mass of matrix (kg)

M : molecular weight (D)

n : number of beads (-)

P_e : partition coefficient (-)

q : concentration of the enzyme on the adsorbent (U/m^3)

q_m : capacity of the adsorbent (U/m^3)

r : radial position in particle (m)

R : radius of particle (m)

t : time (s)

t_d : dimensionless time (-)

v_i : local adsorption velocity ($U/m^3.s$)

V_l : volume liquid phase (m^3)

w : parameter defined in eqn. 16 (-)

x : dimensionless radial position in particle (-)

x_n : parameter defined in eqn. 18 (-)

Greek symbols

α : parameter defined in eqn. 19 (-)

λ : parameter defined in eqn. 26 (-)

ρ_p : density of support (kg/m^3)

τ : dimensionless time (-)

Φ : Thiele modulus defined in eqn. 9c (-)

Θ : parameter defined in eqn. 9c (-)

Ψ : parameter defined in eqn. 9c (-)

ν : parameter defined in eqn. 9d (-)

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CHAPTER 4

ISOLATION OF α-AMYLASE ON CROSSLINKED STARCH

On the interaction of α-amylase with crosslinked starch - Evaluation of process conditions

SUMMARY

The interaction of α-amylase with crosslinked starch is described. The adsorption characteristics are influenced especially by pH and temperature. Adsorption preferentially takes place at 4 ^{0}C. The adsorption behaviour corresponds with the catalytic activity of the enzymes studied. α-Amylase of Bacillus licheniformis which has a broad pH optimum adsorbs over a larger range (pH 5.0-9.0) than the α-amylase from Bacillus subtilis (pH 5.0-7.0). Capacities and Langmuir constants were determined in the relevant pH range. At pH values of 9.0-11.0 the catalytic activity and the adsorption levels drop but the enzyme activity is not irreversibly lost. These conditions are used to recover the enzyme from the matrix. The crosslinked starch matrix is a competitive inhibitor for the enzyme in the enzyme assay. The K_i was determined to be 6-8 mg/ml for the inhibition of B. licheniformis α-amylase. The affinity for soluble starch appears to be approximately 30 times higher than for the matrix. As a result limit dextrin solutions can be used as competitive eluents for the recovery of the enzyme from the adsorbent. A temperature shift from 4 ^{0}C to 70 ^{0}C can be used to recover the enzyme from the adsorbent although this makes the matrix susceptible to biodegradation and enzyme activity is lost. The latter effect can be reduced by adding Ca^{2+} to the system. Sodium chloride and glycerol have an influence on the interaction between α-amylase and the adsorbent. V_{max} of the enzyme and the adsorption levels of α-amylase decrease amongst others as the water activity of the system is lowered. The matrix adsorbs a variety of α-amylases from bacterial and mammalian origin.

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4.1 INTRODUCTION

A downstream process for industrial bulk enzymes usually consists of a cascade of non selective unit operations such as cell separation (filtration or centrifugation), concentration (ultrafiltration), precipitation and/or a drying step [1-3]. As an alternative new process designs are proposed that combine selectivity with ease of recovery by replacing a number of the unit operations mentioned above [3].

It would be interesting to apply affinity interactions in an early phase of the downstream process in such a way that high selectivity can be combined with a continuous process. For large scale affinity separations several demands can be imposed on the adsorbent; i.e. it should be (i) insoluble and chemically stable, (ii) inert, (iii) porous and permeable; (iv) rigid and resistant to compression and (v) inexpensive and reusable [4,5].

For the isolation of polysaccharide degrading enzymes cheap affinity adsorbents, which meet these demands, can be prepared by crosslinking of the substrate thus yielding water insoluble, inert matrices with affinity for the enzymes [3]. In this way crosslinked cellulose, crosslinked pectate and alginate (a substrate analogue for pectate) and crosslinked starch were used to isolate cellulase [6], pectate lyase [7], pectin esterase [7], endo-polygalacturonase [8-11] and α-amylase [6,12-14], respectively.

α-Amylase is an important industrial bulk enzyme for the food processing industry. It finds wide application in the conversion of starch into dextrins, in the baking industry for dough modification and in laundry aids [15]. Most of the industrially produced α-amylases are of bacterial (Bacillus licheniformis and Bacillus subtilis) or fungal (Aspergillus oryzae) origin [16].

An affinity adsorbent for the isolation of bacterial α-amylases was developed by crosslinking potato starch with epichlorohydrin in water-ethanol mixtures under alkaline conditions [12]. A suitable crosslinked starch was synthesized by studying the important paramaters (epichlorohydrin concentration, ethanol-water ratio, sodium chloride-epichlorohydrin ratio, reaction time and temperature) in the crosslinking reaction. In this way a method was developed to produce stable matrices with known properties. The isolation of bacterial α-amylases with these adsorbents has been described [12,13].

In a previous paper possible reaction conditions for the adsorption and desorption reaction were proposed. The adsorption reaction was carried out at 4 ^{0}C, the desorption reaction at 50-70 ^{0}C. Glycerol was added to the process medium to protect the enzyme from inactivation and the adsorbent from

enzymic degradation. Due to the high desorption temperatures required, this process appeared to be only favourable for thermostable α-amylases like the one from B. licheniformis [13].

Successful application of the matrix is hindered by the fact that the desorption step needs a temperature shift which influences the enzyme and the adsorbent stability in a negative way. Generally pH, temperature and ionic strength are considered the most important factors determining the interaction between surfaces and proteins [17,18]. For a satisfactory recovery procedure the influence of these parameters on the equilibrium conditions of the system have to be determined and optimized. This was done for two α-amylase preparations from B. licheniformis and B. subtilis which show different characteristics with respect to their interaction with (crosslinked) starch. These enzymes were also used in the previous work [13].

A system for recovery of α-amylases is described in this paper in which the adsorption and desorption conditions can be manipulated in such a way that the system can be optimally tuned to the process conditions required, the reactor type chosen and the enzyme to be processed.

4.2 MATERIAL AND METHODS

Maxamyl, a heat stable α-amylase from B. licheniformis, Dexlo, an α-amylase from B. subtilis and Mycolase, an α-amylase from Aspergillus oryzae are enzyme preparations of Gist-brocades N.V. (Delft, The Netherlands). The drum dried potato starch powder (Paselli WA 4, ref. no. 20.986) is a product of AVEBE (Veendam, The Netherlands). The dried glucose syrup (limit dextrin fraction) (Mor-Sweet 01921) is a product of CPC/Europe (Utrecht, The Netherlands). Glucose and maltose are from Merck (Darmstadt, FRG). Maltotriose and maltotetraose are from Sigma (St. Louis, USA), maltopentaose is from Boehringer (Mannheim, FRG). Promozyme^{TM} 200 L, a pullulanase preparation from Bacillus species is obtained from Novo Industri A/S (Bagsvaerd/Denmark). Amyloglucosidase (A-3514) from Aspergillus niger, α-amylases (A-3404) from B. licheniformis, (A-0273) from A. oryzae, (A-6380) from Bacillus species, (A-1031) from human saliva, β-amylases (A-7005) from sweet potato, (A-7130) from barley and α-/β-amylase (A-2771) from barley malt are obtained from Sigma Chemical Co., St. Louis, USA. α-Amylase (102814) from pig pancreas and pullulanase (108944) from Aerobacter aerogenes are obtained from Boehringer, Mannheim, FRG.

Preparation of crosslinked potato starch powder

Twenty-five grams of drum-dried starch powder are suspended in 153 ml of ethanol. Subsequently 30.9 ml distilled water, 12.6 ml epichlorohydrin (Merck, Darmstadt, FRG) and 30.85 ml 5M sodium hydroxide are added. The reaction mixture is shaken for 4 h in an orbital incubator (Gallenkamp) at 200 rpm (T = 45 ^{0}C). The reaction is stopped by adding 200 ml 7% (v/v) acetic acid. The reaction mixture is filtered over a glass filter (G2, Schott, Mainz, FRG). The crosslinked powder is washed with 100 ml of ethanol, water (twice), ethanol (twice) and acetone (twice) successively. The powder is air dried. Further details concerning the reaction conditions are given by Rozie et al. [12].

Enzyme activity and protein content

The pH optimum for enzyme activity is determined by incubating the enzyme at saturating substrate conditions at different pH values. For B. licheniformis α-amylase 0.006% (v/v) enzyme solution is incubated in 0.5% (w/v) soluble starch (Merck, Darmstadt, FRG) for 5 min at 30 ^{0}C. The enzyme of B. subtilis is incubated in a concentration of 0.003% (v/v) under the same conditions. The enzyme reaction is stopped by the addition of 0.5 ml of the incubation medium to 1.5 ml sodium carbonate (10% w/v) and cooled on ice. Subsequently reducing end groups are determined as described previously [12] by means of a neocuproin test using an autoanalyzer.

Amyloglucosidase activity is determined in 100 mM sodium acetate pH 6.0 with 0.5% (w/v) soluble starch as the substrate (T = 30 ^{0}C). Pullulanase activity is determined in 100 mM sodium acetate pH 5.0 with 1.1 % (w/v) pullulan as the substrate (T = 30 ^{0}C). D-glucose (α-amylase and amyloglucosidase activity) and maltose (pullulanase activity) were used as the standard. One unit (U) is defined as the amount of enzyme which releases 1 μmol of reducing end groups per minute under these conditions. Units were converted to moles using the values for molecular weight and specific activity as determined previously [13]: for B. licheniformis α-amylase these are 60,000 D and 220 U/mg; for B. subtilis α-amylase 64,000 D and 430 U/mg.

Kinetic experiments are performed as described previously [13]. Inhibition of enzyme activity is measured by addition of inhibitor in the enzyme assay in concentrations as mentioned in the text.

Protein content is determined by using the method of Lowry et al. [19].

Enzyme stability

Enzyme stability is tested by incubation of 10 ml of enzyme solution at

different pH values and temperatures. At regular time intervals 100 μl of sample is taken and diluted in 100 mM sodium acetate pH 6.0. Subsequently the samples are assayed for enzyme activity using an autoanalyzer.

Starch breakdown

Starch powder is assayed for α-amylase degradability at 4 ^{0}C or at 40 ^{0}C by incubation of 20 g/l of crosslinked starch in B. licheniformis α-amylase (3.8×10^{-6} mol/l) for 24 h in a rotating incubator. After centrifugation of the starch 200 μl of the liquid phase is further incubated for 24 h (T = 30 ^{0}C) in 790 μl 100 mM sodium acetate pH 5.0 with the addition of 10 μl 1% (v/v) amyloglucosidase to hydrolyse the polysaccharides present. The samples are analysed for reducing endgroups by means of an automated neocuproin test using an autoanalyzer. D-glucose is used as the standard in these experiments. For details see Rozie et al. [12].

Fractionation of limit dextrin fraction

Fractionation is performed on a Biogel P2 column (Bio-Rad, Richmond, USA) (750 $\times$ 30 mm). Eight hundred milligrams of the limit dextrin fraction are applied in 2 ml distilled water. The column is eluted with distilled water. Flow rate: 0.4 ml/min. Fraction size: 2.5 ml. Fractions are collected and assayed for total sugar content according to Dubois [20].

Fractions are assayed for oligosaccharides by HPLC (SP 8000, Spectraphysics, San José, USA) equipped with an Aminex-HPX-42A column (300 $\times$ 7.8 mm, Bio-Rad, Richmond, USA) and a guard column (50 $\times$ 4.6 mm) packed with a mixture of equivalent amounts of dried AG50W-X4 (H^+, 400 mesh) and AG3-X4A (OH^-, 200-400 mesh, Bio Rad). The analytical column is operated at 85 ^{0}C, the guard column at ambient temperature. Elution rate is 0.5 ml/min. Sugars are detected with an ERM-ERC 7510 refractive index detector at 40 ^{0}C.

Adsorption isotherms

Adsorption isotherms are determined by incubating 0.1 g of crosslinked starch powder for 48 h in 5 ml buffer with different amounts of α-amylase (0 - 3.8×10^{-5} mol/l). The amount of enzyme adsorbed is found by measuring the decrease of enzyme activity in the liquid phase at equilibrium.

Desorption kinetics

Desorption rates are measured by incubating enzyme loaded adsorbent (20 g/l) in the appropriate desorption buffer. The reaction velocity is followed with time by taking samples (20 μl) at regular intervals. During sampling the

powder is temporarily precipitated by means of a centrifuge. The total decrease of the liquid volume due to sampling is less than 5%.

pH dependency

Experiments are performed to investigate the influence of pH on adsorption and biodegradation. The following buffers are used: 100 mM sodium acetate pH 4.0, pH 5.0 and pH 6.0; 50 mM sodium phosphate pH 7.0; 100 mM Tris-HCl pH 8.0; 100 mM Tris-HCl pH 9.0; 50 mM borate-sodium hydroxide pH 9.5 and pH 10.5; 100 mM Glycine-sodium hydroxide pH 11.0 and pH 12.0.

Column chromatography

Several starch degrading enzymes are chromatographed on a column of crosslinked starch which was prepared as described previously [12]. Sample application and washing are performed in 100 mM sodium acetate pH 6.0. Elution of the enzymes is accomplished with a gradient of limit dextrins (0-50 g/l) in 100 mM sodium acetate pH 6.0 or with a pH shift using 50 mM borate-sodium hydroxide buffer pH 10.5. The temperature is 4 ^{0}C during the experiments.

4.3 RESULTS AND DISCUSSION

An important parameter to investigate in the isolation procedure is the enzyme stability during the adsorption and the desorption process. Furthermore, the adsorbent has to be stabilized to make repeated use possible. The effect of pH, temperature and ionic strength on the adsorption and desorption characteristics of α-amylase on crosslinked starch are described. Furthermore, the stabilizing effect of Ca^{2+} on the bacterial α-amylases will be shown.

pH dependency

The relationship between enzyme activity and adsorption behaviour was studied because this may give a direct insight in the involvement of the active site of the enzyme in the adsorption mechanism [10]. Figure 4.1 shows the enzyme activity in terms of V_{max} versus pH. For both enzymes the optimal catalytic activity is obtained at pH 6.0. For the enzyme of B. licheniformis the activity optimum is broader (pH 5.0-8.0) than the optimum of the enzyme of B. subtilis (pH 5.0-6.0). The activity of the B. subtilis enzyme drops to zero at pH 10.0 whereas the enzyme of B. licheniformis retains activity up to pH 11.

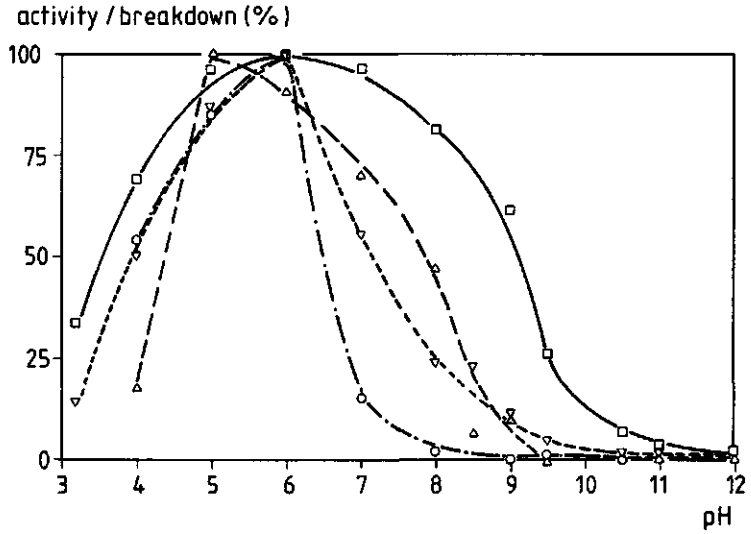

Fig. 4.1 - Enzyme activity of ∝-amylase and breakdown of adsorbent as a function of pH.
B. licheniformis: Enzyme activity: □ . 100% = 360 μmol/minxmg; Breakdown: Δ . Adsorbent concentration: 20 g/l; Enzyme concentration: 3.8 × 10^{-6} mol/l; Maximum breakdown level at assay conditions (100%) = 0.35 g/l.
B. subtilis: Enzyme activity: ∇ . 100% = 600 μmol/minxmg; Breakdown: ○ . Adsorbent concentration: 20 g/l; Enzyme concentration: 3.6 × 10^{-6} mol/l; Maximum breakdown level at assay conditions (100%) = 0.67 g/l.

Adsorption isotherms were determined in a pH range from 5.0 to 11.0 for the enzyme of B. licheniformis. The results are given in Fig. 4.2. The adsorption isotherms were described with the Langmuir equation:

$$C_{sol} = \frac{P_{max} \times C_{liq}}{K_S + C_{liq}} \qquad (1)$$

linearized that is:

$$\frac{1}{C_{sol}} = \frac{K_S}{P_{max}} \times \frac{1}{C_{liq}} + \frac{1}{P_{max}} \qquad (2)$$

In Table 4.1 the adsorption parameters P_{max} and K_S and their reliability intervals are summarized. The capacity of the adsorbent for the B.

licheniformis enzyme does not change in the region of pH 5.0-8.0 (see Table 4.1). Compared to the value obtained at pH 6.0 (11.8 × 10^{-7} mol/l), the Langmuir constant (K_S) increases to 22.6 × 10^{-7} mol/l (at pH 5.0) and 24.5 × 10^{-7} mol/l (at pH 8.0) respectively. This effect shows the decreasing affinity

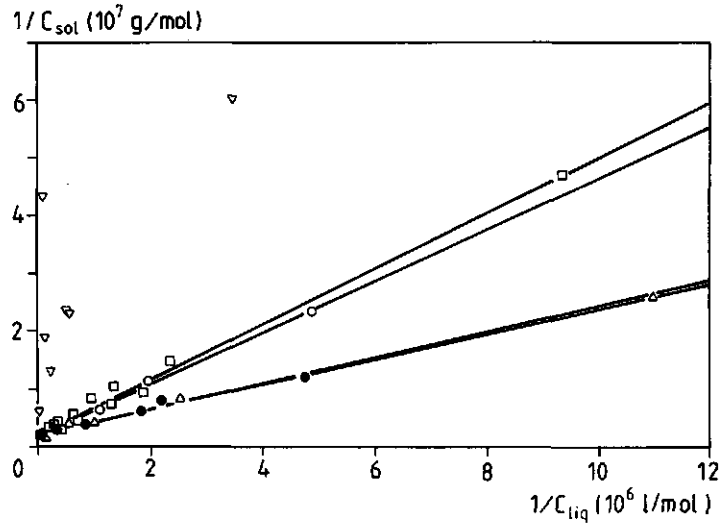

Fig. 4.2 - Linearized adsorption isotherms of B. licheniformis ∝-*amylase on crosslinked starch as a function of pH. T = 4 °C.* □ *, pH 5.0;* △ *, pH 6.0;* ● *, pH 7.0;* ○ *, pH 8.0;* ▽ *, pH 9.0;* ——— *, Langmuir plots with parameters according to Table 4.1.*

of the enzyme for the adsorbent above pH 7.0 and below pH 6.0. From the capacity of the matrix for the enzyme it can be concluded that at pH 6.0 one molecule of B. licheniformis enzyme is associated with approximately 10,500 glucose residues.

At pH 9.0 the data show too much scatter to draw quantitative conclusions. The slope in Fig. 4.2 will increase however and therefore K_S/P_{max} will increase showing a further decrease in interaction between the enzyme and the adsorbent.

When the adsorption isotherms for the enzyme of B. subtilis were determined (see Table 4.1), the value of K_S/P_{max} shows that the interaction between the adsorbent and the enzyme is reduced at pH 7.0 compared to pH 6.0 although it can not be concluded whether this is due to a reduced P_{max} or an increased K_S. The pH related adsorption optimum, however is narrower for this enzyme, which is in line with the shape of its activity profile (Fig. 4.1). At pH 7.0 the V_{max} of the B. subtilis enzyme is reduced compared to the value at pH 6.0. The compliance of the pH related adsorption behaviour with

the activity profile of the enzymes suggests that the interaction between the enzyme and the adsorbent is based on a biospecific interaction [10].

Table 4.1 - pH dependency of the adsorption of B. licheniformis (1) and B. subtilis (2) α-amylases on crosslinked starch. T = 4 ^{0}C.

pH	P_{max} (10^{-7} mol/g)		K_S/P_{max} (g/l)		K_S (10^{-7} mol/l)	
	(1)	(2)	(1)	(2)	(1)	(2)
5	4.7±0.9	-	4.8±0.2	-	22.6±4.3	-
6	5.4±0.5	14±10	2.2±0.1	4.8±0.3	11.8±0.9	67±50
7	4.7±0.9	7±5	2.3±0.2	36±4	10.8±2.2	250±200
8	5.5±0.8	*	4.5±0.2	*	24.5±3.8	*
9	*	*	*	*	*	*

*-: not determined; *: too much scatter in the adsorption data to determine parameters.*

Inactivation constants were determined to find out whether the decrease in enzyme activity and adsorption is due to irreversible enzyme inactivation (Table 4.2). When the enzymes were incubated at 30 ^{0}C for 24 h, no inactivation could be detected within the pH region of 5.0 - 11.0.

The desorption kinetics of B. licheniformis α-amylase from crosslinked starch between pH 6.0 and 12.0 were measured at 4 ^{0}C (Fig. 4.3). The reaction appears not to be completely reversible. At pH 12.0 the maximum desorption level is between 85 and 90% of the amount adsorbed.

Breakdown levels of the matrix were determined as function of pH and enzyme concentration and were found to be particularly pH dependent, decreasing with increasing pH (Fig. 4.4). Figure 4.4 shows that biodegradation of less than 0.1% (w/v) was obtained at 40 ^{0}C at pH values above 9.0. This was confirmed when the adsorbent was suspended in concentrated solutions of B. licheniformis α-amylase (up to 3.8×10^{-5} mol/l).

Table 4.2 - First order inactivation rate constants k (1/s) * 10^5 of ∝-amylases of B. licheniformis and B. subtilis as a function of pH.

pH	Enzyme	
	B. licheniformis	B. subtilis
4.0	22±1	28±1
5.0-11.0	< 0.1	< 0.1
12.0	2.1±0.2	130±10

The pH dependency of biodegradation is also identical to that of the catalytic activity of the enzyme. This fact makes plausible that the interaction between the enzyme and the adsorbent is determined by the active site of the enzymes.

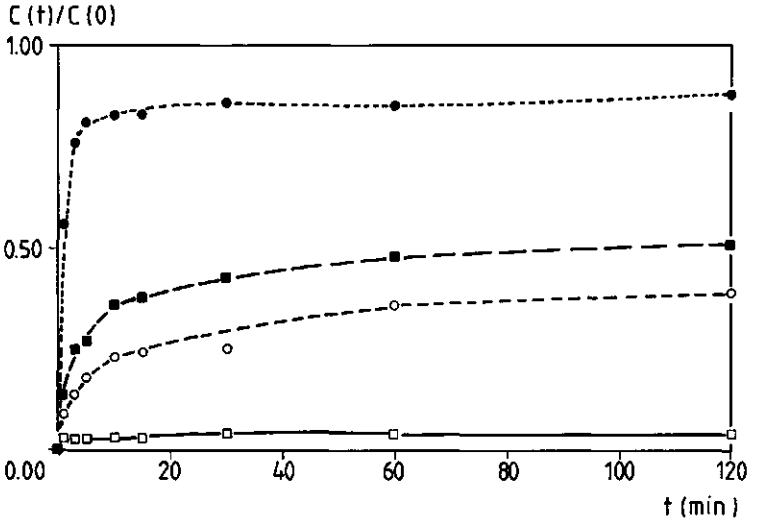

Fig. 4.3 - Desorption of B. licheniformis ∝-amylase from crosslinked starch as function of pH. □ , pH 6.0; ○ , pH 9.0; ■ , pH 11.0; ● , pH 12.0. Adsorbent concentration: 20 mg/ml; Enzyme concentration C(0) : 1.6 × 10^{-7} mol/g; Temperature: 4 ^{0}C.

Because of the observed influence of the temperature on the interaction of the enzyme with the adsorbent [13] a combination of temperature and pH shifts gives the best results in a desorption process.

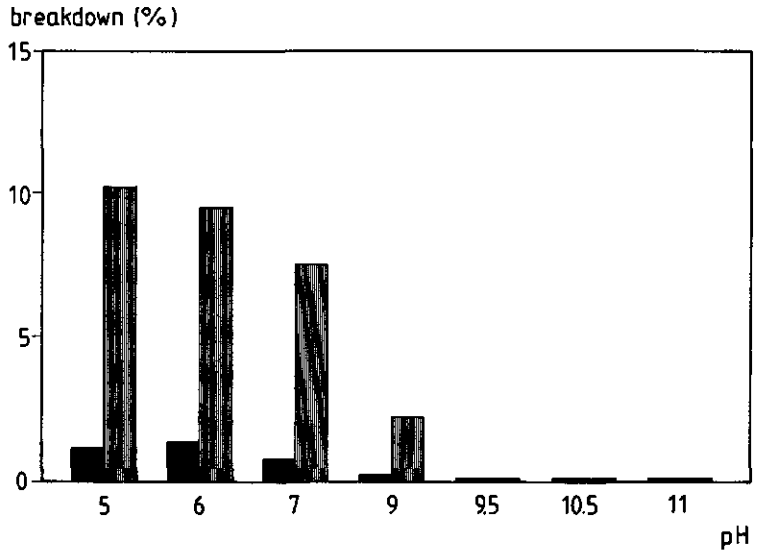

Fig. 4.4 - Breakdown levels of crosslinked starch powder as a function of pH and temperature. ■ . 4 °C; ▥ . 40 °C. Adsorbent concentration: 20 g/l; α-amylase concentration (B. licheniformis): 3.8 × 10^{-6} mol/l. Incubation time: 24 h.

Competitive inhibition of binding

Competitive elution of α-amylase from crosslinked starch with 2M maltose has been previously described [14]. This concentration of competitive eluent would not be feasible in an isolation process for a bulk enzyme. We studied the effect of a commercially produced limit dextrin fraction on the binding of the two α-amylases to the adsorbent. Figure 4.5 shows the adsorption of B. licheniformis and B. subtilis α-amylases to crosslinked starch upon addition of dextrins to the adsorption medium. Under the reaction conditions chosen the binding was zero at a concentration of 15 g/l dextrins in the medium for both enzymes studied. The adsorption of the enzyme of B. subtilis is inhibited at lower concentrations of competitive eluent than that of the one from B. licheniformis. $P_{ad}/P_{ad,0}$ = 0.5 at 2.1 and 7.1 g/l respectively (Fig. 4.5). To obtain more information about the fractions responsible for the inhibition of binding the influence of several commercially obtained oligosaccharides and the

limit dextrin fraction on the enzyme activity were studied. Commercially obtained linear oligosaccharides up to pentamer were not able to inhibit the enzyme activity (not shown). These oligomers are the end product of hydrolysis

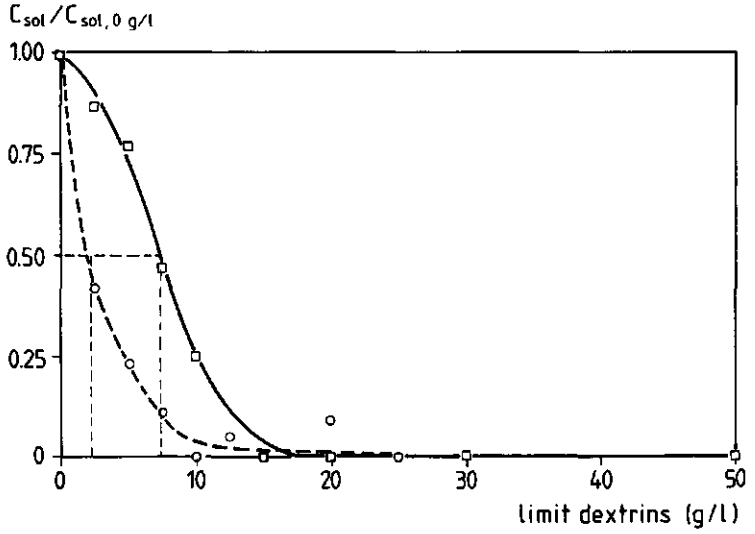

Fig. 4.5 - Inhibition of adsorption of ∝-amylases on crosslinked starch by limit dextrins. Buffer: 100 mM sodium acetate pH 6.0. Adsorbent: 20 g/l; Enzymes: □ , B. licheniformis : C(0): 3.8 × 10^{-6} mol/l; $C_{sol,(0\ g/l)}$: 2.9 × 10^{-6} mol/l; ○ , B. subtilis : C(0): 3.6 × 10^{-6} mol/l; $C_{sol,(0\ g/l)}$: 1.8 × 10^{-6} mol/l.

of starch with this enzyme and product inhibition apparently does not occur [13]. The limit dextrin fraction was fractionated on a Biogel P2 column yielding fractions containing the monomer up to the heptamer (fractions 2-6) and larger oligosaccharides (fraction 1). The fractions were tested for their inhibitory effect in the enzyme assay. The results were evaluated for all the fractions using $[S]/V_0$ versus [S] plots (Dixon plots) [21] and the equation for competitive inhibition :

$$\frac{V_0}{V_{max}} = \frac{[S]}{K_{m(app)} + [S]} \qquad (3)$$

where

$$K_{m(app)} = K_m \left[1 + \frac{[I]}{K_i}\right] \quad (4)$$

Table 4.3 - K_m, $K_{m(app)}$ and V_{max} for the conversion of starch by B. licheniformis α-amylase and inhibition constants (K_i) for several sugar residues.

Inhibitor		V_{max}	K_m, $K_{m(app)}$	K_i
fraction	(g/l)	(μmol/min.mg)	(g/l)	(g/l)
-	-	340±40	0.06±0.02[a]	-
limit dextrin	1	290±40	0.18±0.08	0.5±0.4
fraction 1	0.3	300±40	0.29±0.05	0.08±0.03
fraction 2	0.8	300±40	0.31±0.06	0.20±0.09
adsorbent	20	340±40	0.28±0.02	6±2
adsorbent	40	350±40	0.37±0.02	8±3

[a] *This value represents K_m.*

All concentrations and constants ($K_{m(app)}$ and K_i) are expressed in g/l because the subfractions are not sufficiently characterized. From the fractions obtained after separation on this column only fraction 1 (containing the sugar residues with a degree of polymerization larger than seven) and the fraction containing the penta-, hexa- and heptamer (fraction 2) did inhibit the enzyme (see Table 4.3). The inhibition constant for these fractions is reduced compared to the limit dextrin fraction as a result of the removal of the smaller sugar residues which show no inhibitory effect in the assay (fractions 3-6). The influence of the crosslinked starch on V_{max} and K_m was also investigated by introducing the adsorbent in the enzyme assay (see Table 4.3). The adsorbent is able to inhibit the enzyme. The results show that the inhibition is

competitive (see Fig. 4.6), a straight line parallel to that in the absence of inhibitor is found.

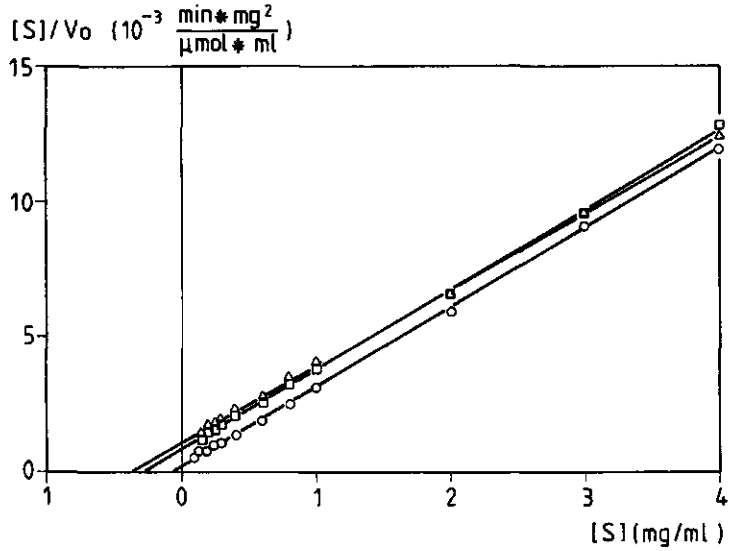

Fig. 4.6 - Inhibition of enzyme activity by crosslinked starch; Assay conditions: see Material and Methods; Inhibitor concentration: ○ *, no inhibitor;* □ *. 20 g/l;* △ *. 40 g/l.*

The fact that the pH optimum of the enzyme activity coincides with that of the binding to the adsorbent and the fact that the adsorbent is a competitive inhibitor for the enzyme indicates that the process of complex formation is due to a functional affinity of the active site of α-amylase for the adsorbent. The K_i for the adsorbent (6 - 8 g/l) was converted to mol/l by dividing this parameter by the molecular mass of the average binding site (10,500 glucose residues) yielding a value of (35-45 ± 20) × 10^{-7} mol/l. These values are of the same order of magnitude as the value of K_S determined for equilibrium adsorption ((11.8 ± 0.9) × 10^{-7} mol/l, see Table 4.1)). The fact that the value of K_i is larger than K_S might be due to the fact that during the inhibition assay not all the binding sites on the adsorbent can be reached by the enzyme due to the diffusion resistance of the matrix. From these results it may be concluded that the affinity of the enzyme for the matrix is in fact approximately 30 times lower instead of 100-130 times lower than that for the soluble substrate.

Ionic strength and water activity

In previous studies we found the adsorption characteristics of B.

licheniformis α-amylase to be independent of ionic strength. This appears to be true in an ionic strength region up to 1 M sodium chloride. When the salt concentration is raised further to 5 M sodium chloride an effect on the adsorption behaviour of the enzyme is observed. Adsorption isotherms were

Table 4.4 - Adsorption isotherms of B. licheniformis α-amylase on crosslinked starch as a function of temperature and sodium chloride concentration.

NaCl (M)	T (^{0}C)	P_{max} (10^{-7} mol/g)	$K_S/Pmax$ (g/l)	K_S (10^{-7} mol/l)
0	4	5.4±0.5	2.2±0.1	11.8±0.9
1		3.1±0.5	3.7±0.3	12±2
2		2.7±0.5	3.9±0.4	11±2
3		1.8±0.2	4.8±0.3	9±1
0	0	5.9±0.5	2.0±0.2	12±1
	10	7.2±0.7	3.2±0.3	23±5
	20	4.0±0.6	7.3±0.4	29±5
	30	3.3±0.5	8.6±0.4	28±5
	40	2.2±0.3	14.1±0.6	31±6

determined at varying sodium chloride concentrations (Table 4.4). Generally the adsorption levels at 4 M and 5 M sodium chloride do not exceed 0.7 × 10^{-7} mol/g. The results are not given because the correlation in the adsorption data is low. It appears that the decreasing interaction between the adsorbent and the enzyme is particularly due to a decreasing capacity of the matrix for the enzyme. The Langmuir constant K_S is approximately the same at all salt concentrations showing that the interaction between the enzyme and the adsorbent in itself is unchanged. The findings are different to the effect

observed as a result of pH increase, where beyond pH 6.0 K_s increases with reduction of V_{max} while the values for P_{max} are constant (Table 4.1 and Fig 4.1). While the changing interaction as a function of pH may be explained by a conformational change in the active site of the enzyme a decreasing availability of binding sites at increased ionic strength may explain the observed reduction of P_{max} under these conditions.

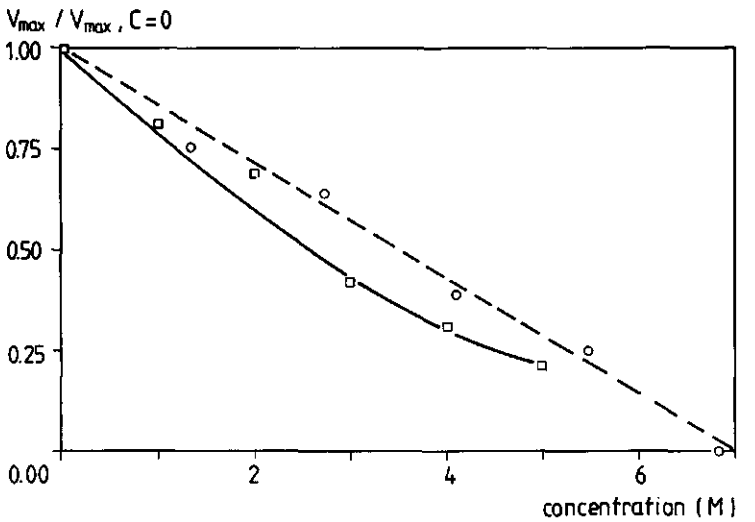

Fig. 4.7 - Enzyme activity (V_{max}) as function of glycerol and sodium chloride concentration; □ , sodium chloride; ○ , glycerol; Buffer: 100 mM sodium acetate pH 6.0; Full scale (1.0): V_{max} = 360 μmol/min.mg.

In earlier studies we found desorption to be facilitated if glycerol is added to the reaction medium [13] in concentrations of 20% (v/v) and up. As glycerol is also claimed to be a competitive inhibitor of α-amylase [22] we determined whether this is true under these conditions. The V_{max} of the B. licheniformis α-amylase as a function of salt and glycerol concentration was determined under saturating substrate conditions (Fig. 4.7). It appears that V_{max} is reduced both in experiments with high salt concentrations and with high concentrations of glycerol. These results show that the effect of glycerol on the adsorption and desorption characteristics of the system as described previously [13] is not solely due to a competitive inhibitory effect [22]. Furthermore, these results combined with the results from the competition experiments, suggest that the elution of α-amylase from crosslinked starch by 2

M maltose [14] may in fact not be due to competitive effects either but merely to a change of other properties of the desorption buffer. A resemblance between high glycerol concentrations (from 20% (v/v) and up) and high salt concentrations is that the water activity of these systems is influenced [23-25]. In this respect it must be noted that withdrawal of water from the system may have an effect on the enzyme as well as on the substrate. Conformational changes in amylose and amylopectin structure due to changes in the ionic strength of the medium are known although they are the most predominant in alkaline solutions [26]. High salt concentrations in the medium normally yield a decrease in viscosity of the medium. The fact that at high salt concentrations the affinity of the enzyme for the adsorbent remains unchanged may implicate that the changes are rather due to changes on the substrate than on the active site of the enzyme. However, one has to keep in mind that other parameters (e.g. the dielectric constant) may influence the interaction as well.

Table 4.5 - Inactivation constants k (1/s) * 10^5 of B. licheniformis (1) and B. subtilis (2) α-amylases at various temperatures.

$CaCl_2$	Enzyme	Temperature (^{0}C)			
(mM)		60	70	80	90
0	(1)	< 0.1	12±1	80±6	190±10
	(2)	9.5±0.4	150±10	960±50	2500±100
0.5	(1)	-	-	-	22±1
1	(1)	-	-	-	12±1
5	(1)	-	-	-	2.2±0.4
7.5	(1)	-	-	-	1.8±0.4
10	(1)	< 0.1	< 0.1	< 0.1	2.5±0.4
	(2)	< 0.1	2.5±0.3	8.0±0.4	450±40
50	(1)	< 0.1	< 0.1	< 0.1	6.0±0.4
	(2)	< 0.1	5.0±0.3	90±5	500±40
100	(1)	< 0.1	< 0.1	< 0.1	14±1
	(2)	< 0.1	7.0±0.5	110±5	550±40

-: not determined.

Temperature dependency

A disadvantage of operating the extraction process of α-amylases with the adsorbent at temperatures above 4 ^{0}C is the increasing biodegradability of the matrix with temperature. At pH 6.0 the breakdown of the adsorbent goes gradually up from 0.9 % (w/v) to 15 %(w/v) when adsorption isotherms were determined for the enzyme of B. licheniformis within a temperature range of 0 ^{0}C to 40 ^{0}C. Under all these conditions the breakdown levels were not dependent on the enzyme concentration in the incubation medium. The interaction of the enzyme with the matrix decreases with temperature as shown earlier [13]. At 4 ^{0}C and 10 ^{0}C saturating type isotherms were obtained. In the temperature range between 20 ^{0}C and 40 ^{0}C a decrease in affinity is observed (Table 4.4). The capacity of the matrix can not be deduced directly from the adsorption data because saturation is not reached under these conditions. Furthermore the adsorption parameters are hard to interpret because of the relatively high amount of biodegradation in the samples under these conditions. Biodegradation influences the equilibrium state of the system because of the release of soluble competitive inhibitors in the incubation medium.

Calcium dependency

Some α-amylases are known to be stabilized by the addition of calcium ions to the incubation medium [27]. This was shown to be particularly the case for the thermostability of α-amylase of B. subtilis. Recently, for the heat stable α-amylase of B. licheniformis calcium dependency was also described [28]. This α-amylase shows enhanced stability at temperatures above 60 ^{0}C when Ca^{2+} is added to the medium. Inactivation constants of the two enzymes show that there is a stability optimum for both enzymes below 10 mM calcium chloride in a 100 mM sodium acetate buffer pH 6.0 (Table 4.5). Above 10 mM calcium chloride there is again an increase in the inactivation rate. No inactivation could be detected in the B. licheniformis samples at 70 ^{0}C and 80 ^{0}C for at least 24 h in the presence of 10 mM calcium chloride. At 90 ^{0}C inactivation was observed. At this temperature the enzyme is stabilized by a factor of hundred by the addition of 7.5 mM calcium chloride to the medium (Table 4.5). These results compare well with other studies in which 5 mM calcium chloride was found to be the optimal concentration [28].

When isolating α-amylases from the adsorbent by a temperature shift it is important to protect the enzyme against denaturation. This recovery procedure has the advantage that the process medium is the same during the whole

process. Although this method for recovery was indicated in a previous paper [13] it was not a very realistic option for the relatively heat unstable ∝-amylase from B. subtilis. Addition of calcium chloride to the process medium stabilises this enzyme resulting in no detectable inactivation of this enzyme after 24 h at 60 ^{0}C (see Table 4.5).

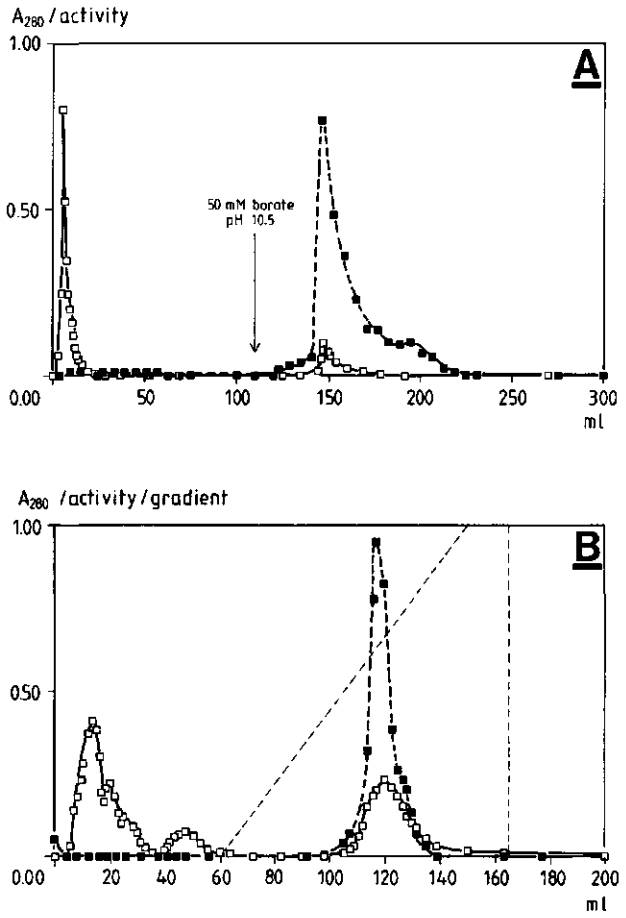

Fig. 4.8 - A Isolation of B. subtilis ∝-amylase on crosslinked starch using a pH shift. A_{280}: □ ; scale (1.0) = 0.4; Activity: ■ ; scale (1.0) = 1.8 × 10^{-6} mol/l. Initial pH: 6.0.

B Isolation of B. licheniformis ∝-amylase on crosslinked starch using a limit dextrin gradient (0-50 g/l). A_{280}: □ ; scale (1.0) = 0.5; Activity: ■ ; scale (1.0) = 1.2 × 10^{-6} mol/l. pH: 6.0.

The adsorbent stability however is lowered at process temperatures of 20 ^{0}C and higher (See section "Temperature dependency"). To obtain protection of the adsorbent at higher temperatures glycerol can be added [13] to the

medium. This is disadvantageous because it implicates different adsorption and desorption media during the isolation process.

Column chromatography

The matrix was used to study the possibility to purify α-amylases from different origins by column chromatography. Figure 4.8 shows the isolation of the α-amylases of B. licheniformis and B. subtilis from commercial enzyme samples on a column of crosslinked starch. The enzymes were eluted by a limit dextrin gradient and a pH shift respectively. The specific activity of the purified enzymes was 440 U/mg for the α-amylase from B. subtilis and 220 U/mg for the one from B. licheniformis. This is in good agreement with the results obtained from a desorption step by a temperature shift [13].

Table 4.6 shows some figures for the isolation of different α-amylases and other polysaccharide degrading enzymes using this matrix. The reaction conditions were not optimized for the different enzymes and adsorption was performed at pH 6.0 in all cases. From Table 4.6 it can be concluded that fungal α-amylases do not adsorb to the matrix while their pH optimum is known to be around pH 5.0-6.0 [27]. Bacterial and mammalian α-amylases are readily and preferentially adsorbed to the matrix.

Furthermore it is shown that the α-amylases of B. subtilis and Bacillus species (Sigma) are purified to the same extent on the adsorbent. The human saliva α-amylase was purified by a factor of 7 from the commercial preparation.

The β-amylase of sweet potato is also adsorbed by the adsorbent in considerable amounts. No adsorption of amyloglucosidase and pullulanase was observed under the reaction conditions chosen.

4.4 CONCLUSIONS

The catalytic optima of the α-amylases investigated correspond to their adsorption behaviour on crosslinked starch. Affinity for and biodegradation of the adsorbent by the enzyme are a function of its activity profile. Furthermore the adsorbent is a competitive inhibitor for α-amylase. These results show that the interaction between the enzyme and adsorbent is biospecific and that the active site of the enzyme is involved in the adsorption to its modified substrate.

The system offers good opportunities to stabilise the enzyme and the adsorbent and to adsorb and desorb the enzyme from the matrix. The influence

Table 4.6 - Adsorption of polysaccharide degrading enzymes to crosslinked starch. Adsorbent concentration: 20g/l.

Enzyme	P_0[1]			P_{ad}[1]		adsorbed fraction
(Organism)	(U/ml)	(mg/ml)	(U/mg)	(U/ml)	(mg/ml)	(U/mg)
α-amylase						
1.A. oryzae Mycolase	14	.21	67	14	.20	-
2.A. oryzae (Sigma)	11	.19	58	10.2	.21	-
3.B. licheniformis Maxamyl	57	.36	158	13	.18	245
4.B. licheniformis (Sigma)	38	.65	59	6	.54	290
5.B. subtilis Dexlo	61	.25	244	13	.13	400
6.Bacillus species (Sigma)	120	.39	310	33	.16	380
7.Pig pancreas	5.4	.26	20.8	1.3	.04	18.7
8.Human saliva	3.6	.18	20	0.8	.16	140
α-amylase/β-amylase						
9.Barley malt	2	.06	33	1.1	.04	45
β-amylase						
10.Sweet potato	103	.17	605	55	.10	685
11.Barley malt	3.2	.35	9	2.8	.3	8
amyloglucosidase/ pullulanase						
12.Amyloglucosidase	4	.64	6.3	3.9	.64	-
13.Pullulanase[2] Aerobacter aerogenes	2.2	.07	31	2	.07	-
14.Pullulanase[2] Bacillus species Promozym	2	.14	14.3	1.9	.13	-

[1]: P_0: enzyme concentration in liquid phase at t = 0 (U/ml); P_{ad}: enzyme concentration in liquid phase at equilibrium (U/ml); [2]: pullulanase activity; -: not calculated due to low adsorption level.

of pH, temperature, calcium chloride and ionic strength are described.

Ideal adsorption characteristics are obtained at the optimum pH (6.0-7.0) and low temperatures (below 4 ^{0}C). This gives the maximum degree of interaction combined with optimal protection of the adsorbent against biodegradation. Desorption should be performed at increased temperatures (above 50 ^{0}C) combined with a pH shift (pH 11.0-12.0) to protect the matrix against biodegradation. The enzyme can be stabilised by the addition of 10 mM calcium chloride.

The effect of ionic strength is negligible in the purification process. Only at increased ionic strength (above 1 M sodium chloride) a decrease in adsorption capacity is observed. Addition of glycerol in the desorption step may improve the process, yielding higher desorption levels and improved enzyme stability [13].

Crosslinked starch can be used to isolate a number of α-amylases. Depending on the specific enzyme pH and temperature influences have to be investigated and optimized. It has been shown that the matrix has good properties for the development of affinity separation processes for a number of α-amylases.

Acknowledgements

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NOMENCLATURE

$C(0)$: enzyme concentration at t = 0 (mol/l)
$C(t)$: enzyme concentration at t = t (mol/l)
C_{liq} : enzyme concentration in liquid phase at equilibrium (mol/l)
C_{sol} : enzyme concentration in solid phase at equilibrium (mol/g)
[E] : enzyme concentration (g/l)
[EI] : enzyme/inhibitor complex concentration (g/l)
[I] : inhibitor concentration (g/l)
k : first order inactivation rate constant (1/s)
K_a : affinity constant (l/g)
K_i : inhibition constant (g/l)
K_m : Michaelis Menten constant (g/l)
$K_{m(app)}$: apparent Michaelis Menten constant (g/l)
K_s : Langmuir constant (mol/l)
P_{ad} : enzyme concentration in liquid phase at equilibrium (U/ml)

P_{max} : capacity of adsorbent (mol/g)

P_0 : enzyme concentration in liquid phase at t = 0 (U/ml)

[S] : substrate concentration (g/l)

t : time (min)

U : unit

V_0 : initial turnover rate (μmol/min.mg)

V_{max} : maximum turnover rate (μmol/min.mg)

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CHAPTER 5

ISOLATION OF α-AMYLASE ON CROSSLINKED STARCH

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SUMMARY

The isolation and purification of α-amylase from an industrial enzyme sample is described using crosslinked starch powder as an affinity adsorbent. The reaction conditions were determined with regard to the stability of the adsorbent, the stability of the enzyme and the capacity of the adsorbent for the enzyme.

The adsorbent was used in a repeated adsorption/desorption process to evaluate the binding capacity and the stability of the adsorbents in continuous processes. The adsorption kinetics of the matrix is improved due to the fact that during the repeated process the diffusion resistance of the matrix is decreasing. This effect is accompanied by an increase of the capacity of the adsorbent for the enzyme, yielding adsorption levels up to 0.5 mg protein per mg adsorbent. The matrix itself is slowly degraded, but no decline of the adsorption levels was observed during 90 cycles under the experimental conditions used. The life time of the matrix is estimated to be 140 repeated runs. Approximately 10 to 40 kg of pure α-amylase can be obtained with 1 kg of adsorbent. This may yield an economically attractive purification process because the adsorbent is cheap and easy to prepare, as it consists of starch crosslinked with epichlorohydrin.

Submitted for publication.

5.1 INTRODUCTION

Introduction of affinity chromatography in an early stage of the downstream process can be an attractive method to isolate proteins on a large scale [1-6].

The major drawback for application of affinity chromatography in an early phase of the isolation process (i.e. directly on the fermentation broth) is twofold: packed bed chromatography can easily lead to clogging and fouling of the column and adsorbents often are very expensive.

The first problem can be overcome by using continuous adsorption systems with affinity ligands coupled to open and porous matrices or with soluble affinity adsorbents [4,7-11]. The majority of these studies use affinity ultrafiltration techniques [8-12].

The second problem can be overcome by the development of an affinity adsorbent that is cheap or that can be used for an extended period of time. An affinity adsorbent with these properties was designed for the isolation and purification of endo-polygalacturonase [7,13]. This method is based on the interaction of the enzyme with alginate, the substrate analogue for the natural substrate pectate [14]. For the isolation of α-amylase an affinity adsorbent, based on enzyme/substrate interactions, was developed also [15]. Starch, crosslinked with epichlorohydrin, is stable against biodegradation by α-amylase and has a good binding capacity for the enzyme [15-17]. Adsorption and desorption conditions for this system depend on several parameters. Adsorption is preferably conducted at 4 ^{0}C. At this temperature the adsorption levels are high whereas breakdown of the adsorbent by the enzyme is minimal [18]. In addition there appears to be a relation between the adsorption characteristics and the activity optimum of bacterial α-amylases. Maximum adsorption is found at pH 5.0-6.0, the optimal pH for catalytic activity. It was shown that the interaction between the matrix and the enzyme is biospecific. This allows also recovery of the enzyme from the matrix using a competitive eluent, for instance a limit dextrin fraction [19].

A major obstruction for the use of the powders in an isolation process is the very low reaction velocity of the adsorption reaction [18]. Although the capacities of the crosslinked products under equilibrium conditions (5.5 - 10.0 × 10^{-7} mol/g) offer a good potential for the isolation of the enzyme, no substantial binding of α-amylase was observed in the first six hours of the the reaction [18,19].

It is the objective of this study to improve the adsorption characteristics of crosslinked starch. The adsorbent has therefore been characterised with

respect to its kinetic behaviour, adsorption capacity, biodegradation by α-amylase and its usefulness in a repeated adsorption/desorption process.

5.2 MATERIAL AND METHODS

Materials

Maxamyl, a heat stable α-amylase from Bacillus licheniformis, is an enzyme preparation of Gist-brocades N.V. (Delft, The Netherlands). The drum dried potato starch powder (Paselli WA 4, ref. no. 20.986) is a product of AVEBE (Veendam, The Netherlands).

Determination of enzyme activity and protein content

α-Amylase activity is determined by an automated neocuproin test using an autoanalyzer (Skalar, Breda, The Netherlands) as described previously [15]. Protein content is determined by using the method according to Lowry [20]. One unit (U) is defined as the amount of enzyme which releases 1 μmol of reducing end groups per minute at pH 6.0 and 30 ^{0}C. Units can be converted to moles using the values for molecular weight (60,000 D) and specific acitivity (220 U/mg) [18].

Preparation of crosslinked potato starch powder

25 g of drum dried starch powder is suspended in 153 ml of ethanol. Subsequently 30.9 ml distilled water, 12.6 ml epichlorohydrin (Merck, Darmstadt, FRG) and 30.85 ml 5 M sodium hydroxide are added. The reaction mixture is shaken for 4 h in an orbital incubator (Gallenkamp) at 200 rev/min (T = 45 ^{0}C). The reaction is stopped by adding 200 ml 7% (v/v) acetic acid. The reaction mixture is filtered over a glass filter (G2, Schott, Mainz, FRG). The crosslinked powder is washed with 100 ml of ethanol, water (twice), ethanol (twice) and acetone (twice), respectively. The powder is air dried. The crosslinking reaction is also performed using a ground powder. To this end the starch powder is ground in a Retsch grinder with a sieve size of 0.08 mm. The reaction conditions for crosslinking are the same as described above.

Particle size reduction

25 g of crosslinked starch is ground in a Retsch grinding device with a sieve size of 0.08 mm. The powder obtained in this way has a maximum particle size of 0.08 mm and was used for binding studies. When used the average particle size will increase (though less than 50%) due to swelling in

aqueous media.

Powder size distribution

Particle size distributions are determined with a Malvern 3300 particle sizer. 2 mg of powder are suspended in 15 ml ethanol (p.a., Merk, Darmstadt, FRG). The upper detection limit in this system is 564 μm.

Particle size distributions are also determined by measuring the diameter of a population of approximately 200 particles using a binocular microscope with measuring device.

Starch breakdown

Starch powders are assayed for biodegradability as described previously [15] by incubating appropriate amounts of crosslinked starch (20 g/l) with α-amylase (50 $\times$ 10^3 U/l). The samples are analysed for reducing end groups by means of an automated neocuproin test using an autoanalyzer (Skalar). D-glucose was used as the standard.

Adsorption isotherms

Adsorption isotherms are determined by incubating 0.1 g of crosslinked starch powder in 5 ml 100 mM sodium acetate pH 6.0 with varying amounts of α-amylase (0 - 5000 $\times$ 10^3 U/l). The amount of adsorbed enzyme is found by measuring the decrease of enzyme activity in the liquid phase at equilibrium.

Adsorption kinetics

Usually 0.03 - 0.04 g of crosslinked starch is put in a 2.0 ml reaction vessel with cap. 1.5 ml of an enzyme solution (50 $\times$ 10^3 - 1000 $\times$ 10^3 U/l) is added to the vessel at t = 0. The tube is placed in a rotating incubator. For sampling the tubes are centrifuged in an eppendorf centrifuge to precipitate the powder (about 5 sec). Then 20 μl of the supernatant is taken and diluted to assay for enzyme activity. The change in the liquid volume is less than 10% in these experiments.

Desorption kinetics

Desorption is accomplished in the adsorption buffer by the addition of 20% (v/v) glycerol (further called desorption buffer). 0.03 g of crosslinked starch is suspended in 1.5 ml of desorption buffer. The reaction velocity is followed with time taking samples (20μl). The sampling was done as described under "Adsorption kinetics". The total change in the liquid volume is less than 10% in these experiments.

Repeated Adsorption/Desorption experiments

Crosslinked starch (24-33 g/l) is suspended in a perspex column (i.d.: 0.05 m; h: 0.48 m) provided with a cooling mantle and with a glass filter at the bottom (cutoff: 90-140 μm). At the start of the experiment α-amylase (Maxamyl, 50 × 10^3 - 1000 × 10^3 U/l) is pumped into the column (T = 4 ^{0}C). The adsorption reaction is stopped after 100 min and the supernatant is pumped out of the column. Subsequently the matrix is washed in cold 100 mM sodium acetate pH 6.0 (T = 4 ^{0}C, 5 min). The enzyme is desorbed by the addition of 100 mM glycine/sodium hydroxide pH 11.7 (T = 4 ^{0}C, 45 min). Subsequently the matrix is regenerated for adsorption by washing with 100 mM sodium acetate pH 6.0 (T = 4 ^{0}C, 10 min) and the process is started again. After each step samples are taken to determine the enzyme activity, protein content and starch breakdown.

Gel filtration chromatography

Gel filtration experiments were carried out at room temperature. The column (dimensions 12 × 800 mm) is filled with the adsorbent which is swollen in 100 mM sodium acetate at pH 6.0 for 1 h. An activated matrix is obtained after incubation of 160 g of adsorbent in 800 ml 100 mM sodium acetate buffer pH 6.0 with Maxamyl (50 × 10^3 U/l). After incubation the enzyme is eluted from the matrix at 70 ^{0}C by repeated incubation (three times) of the adsorbent in 300 ml sodium acetate buffer pH 6.0 with the addition of 20% (v/v) glycerol. Subsequently the adsorbent is used in gel filtration chromatography in the same column. Flow: 0.6 ml/min. As standards Dextran Blue (MW >> 450 kD), ferritin (450 kD), BSA (68 kD), ovalbumin (43 kD), chymotrypsinogen (25 kD), lysozyme (25 kD), bacitracine (1.4 kD), tyrosine 777 D and sodium azide (63 D) were used.

5.3 THEORY

Previously [18,19] it was shown that the adsorption velocity of α-amylase on crosslinked starch is very slow although at equilibrium adsorption capacities of 7.3 × 10^6 up to 13.3 × 10^6 U/kg were found [19]. The adsorption velocity of an adsorbate on a porous affinity matrix is generally determined by liquid film transport, pore diffusion, surface diffusion and kinetics of complex formation, their relative importance being characterized by the following dimensionless groups [21].

The relative importance of the film controlled mass transfer and the adsorption kinetics with respect to the pore diffusion is given by the Biot number (Bi) and the Thiele modulus (Φ) respectively:

$$Bi = \frac{k_l \times R}{D_{eff} \times P_e}; \qquad (1)$$

$$\Phi^2 = \frac{R^2 \times k_i}{D_{eff}}; \qquad (2)$$

The relative importance of surface diffusion to pore diffusion is characterized by the parameter λ :

$$\lambda = \frac{D_s \times q_m}{D_p \times c_{b,0}} \qquad (3)$$

The parameter ν gives the relative importance of the adsorption velocity of complex formation compared to the liquid film mass transport:

$$\nu = \frac{k_i \times R}{k_l} \qquad (4)$$

From these groups it can be concluded that adsorption kinetics can be improved by reduction of the particle size (R), by improved mass transfer (k_l, D_{eff}, D_s) or by accelerated reaction kinetics (k_i). Changing only one of these variables will not always necessarily accelerate the reaction velocity in case another parameter is controlling the process. It can be assumed that the slow adsorption velocity in the process under study [15] is amongst others due to mass transfer problems. In general diffusion is rate determining in adsorption of proteins on porous matrices [7,21,22,23]. In that case Φ and Bi are much larger than 1. The reduction of the particle size and the reduction of the diffusion resistance become then of major importance. The effect of particle size distribution and mass transfer on the adsorption reaction have therefore been analysed further.

5.4 RESULTS

Adsorption isotherms

Adsorption equilibria are described with the Langmuir equation:

$$q = \frac{q_m \times c}{K_s + c} \qquad (5)$$

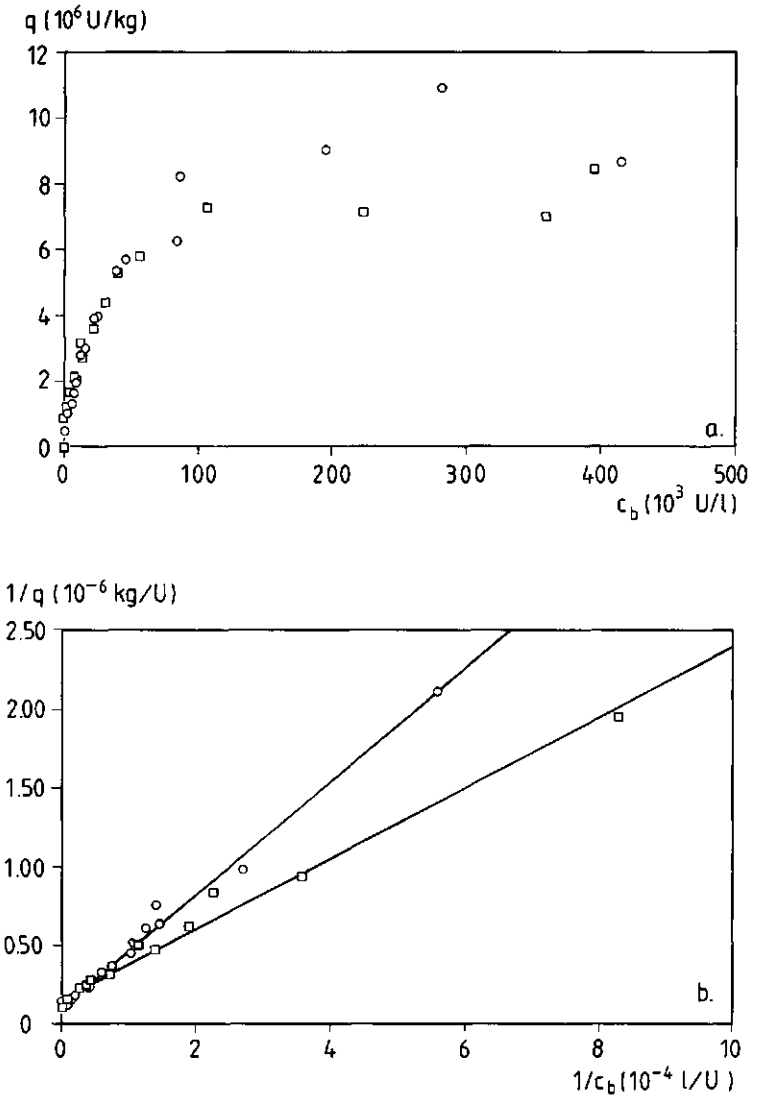

Fig. 5.1 - (a) Adsorption isotherm of alpha-amylase on crosslinked starch. □ : crosslinked starch; ○ : ground crosslinked starch; Buffer: 100 mM sodium acetate pH 6.0; T = 4 °C. (b) Linearized isotherm. □ : crosslinked starch; ○ : ground crosslinked starch.

Figure 5.1 and Table 5.1 show the adsorption isotherms and the calculated capacities and Langmuir constants for the crosslinked starch and the ground crosslinked starch. It appears that the adsorbents have comparable properties concerning their equilibrium behaviour. However, the capacity of the ground crosslinked starch is about 50% larger compared to the crosslinked starch. The results also show a decreased affinity of the enzyme towards the ground crosslinked starch when compared to the crosslinked starch. The breakdown of the ground crosslinked starch by the enzyme is larger at all conditions examined, resulting in higher amounts of soluble oligosaccharides in the experiments (Table 5.2). Oligosaccharides may act as competitive inhibitors [19].

Table 5.1 - Adsorption isotherms of crosslinked starch.

matrix	q_m (10^6 U/kg)	K_S/q_m (10^{-3} l/kg)	K_S (10^3 U/l)
crosslinked starch	6.4±0.8	2.1±0.2	13.3±2.1
ground crosslinked starch	9.6±1.1	3.4±0.2	32.7±5.0

In the concentration range as shown in Table 5.2 this will result in lower adsorption levels at equilibrium. This can explain the apparent lower affinity constant K_S, shown in Table 5.1. The matrices can be stabilized during desorption by using glycerol [18] or by applying a pH shift to pH 11.0 where the enzyme activity is reduced [19].

Adsorption kinetics

Figure 5.2(a) shows the adsorption of a Maxamyl sample (50×10^3 U/l) to crosslinked starch and the ground crosslinked starch. As can be seen from Fig. 5.2(a) only 10-15% of the enzyme activity is adsorbed by the crosslinked powder whereas the ground crosslinked starch absorbs 80% of the enzyme

activity. The adsorption velocity is accelerated as a result of the particle size reduction.

Table 5.2 - Biodegradation of crosslinked starch - measured amount of soluble oligosaccharides.

powder type	biodegradation					
	adsorption buffer				desorption buffer	
	4 ^{0}C		40 ^{0}C		40 ^{0}C	
	g/l	(%)	g/l	(%)	g/l	(%)
crosslinked starch	0.15	0.9	0.58	2.9	0.32	1.6
ground crosslinked starch	0.22	1.1	1.06	5.3	0.42	2.1

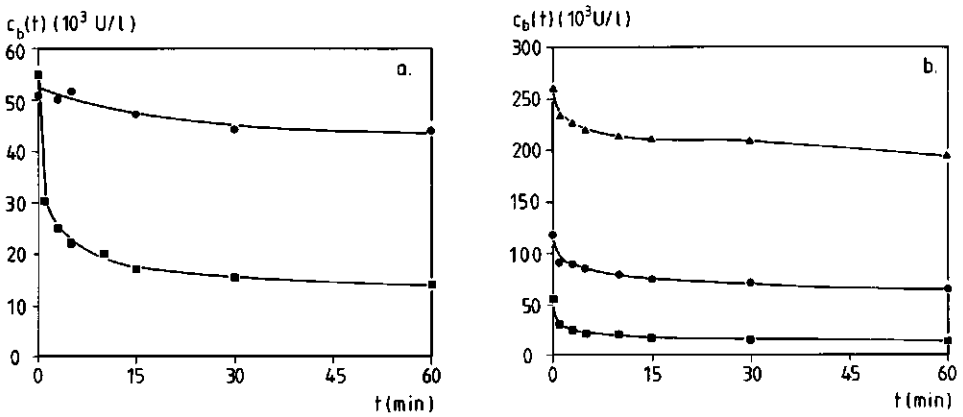

Fig. 5.2 - (a) Adsorption kinetics of alpha-amylase on crosslinked starch. ● : crosslinked starch; ■ : ground crosslinked starch. $c_{b,0}$ = 50 × 10^3 U/l; adsorbent: 20 g/l; Buffer: 100 mM sodium acetate pH 6.0; T = 4 ^{0}C.
(b) Adsorption kinetics of alpha-amylase on ground crosslinked starch, variation of initial enzyme concentration. ■ : $c_{b,0}$ = 50 × 10^3 U/l; ● : $c_{b,0}$ = 120 × 10^3 U/l; ▲ : $c_{b,0}$ = 260 × 10^3 mol/l; adsorbent: 20 g/l; Buffer: 100 mM sodium acetate pH 6.0; T = 4 ^{0}C.

Figure 5.2(b) shows the reaction kinetics of the adsorption reaction for different initial concentrations of enzyme and a constant powder concentration (ground crosslinked starch). Independent of the initial concentration of enzyme a fixed amount of enzyme of approximately 40×10^3 U/l is absorbed in the first 10 min. The adsorption levels predicted by the adsorption isotherm (see Table 5.1, Fig. 5.2(b)) are not reached within this time span.

The mass transfer mechanism has to be determined by D_{eff}, k_i and/or k_l. When particle pore diffusion would be rate limiting a dependency of the adsorption velocity on the bulk liquid concentration is expected for the time scale studied [21]. This is certainly not the case.

The results for the initial period might be explained by a limitation of external mass transport (k_l) or the kinetics of complex formation (k_i). The following criterion for the case that k_l is rate limiting is given [24] by:

$$\frac{V_l(c_{b,0} - c_b(t))}{3m_p \times q_m} << 1 \qquad (6)$$

In that case the value of k_l can be derived from [24]:

$$1 - \frac{c_b(t)}{c_{b,0}} = \frac{3m_p}{\rho_p \times V_l}\left(\frac{k_l \times t}{R}\right) \qquad (7)$$

Equation 7 shows that the adsorption should increase with increasing $c_{b,0}$. This does not occur which indicates that k_l is not the rate limiting factor. Assuming that k_i determines the initial part of the adsorption curve then an estimate of the binding constant of the enzyme to the adsorbent can been made assuming first order kinetics for the complexation reaction and k_i as the rate limiting factor. The calculations reveal a value of $k_i > 1.6 \times 10^{-2}$/s. k_l normally has values of 10^{-5}-10^{-6} m/s for proteins. The value of the dimensionless group $k_i.R/k_l$ (eqn. (4)) is 0.064-0.64. This shows that it is still possible that the experimental adsorption data have to be described by combined reaction kinetics and film mass transfer effects [25]. However the adsorption then should be dependent of $c_{b,0}$ and this is not the case. This contradiction together with the slow saturation part of the curves in Fig. 5.2(b) makes it plausible that another mechanism not included in the eqns. 1-7 is of importance. This will be discussed further in the paragraph concerning repeated adsorption/ desorption experiments.

Desorption kinetics

The desorption of the enzyme from the crosslinked starch can be accomplished by suspending a loaded powder in 100 mM sodium acetate pH 6.0 with 20% (v/v) glycerol at 70 ^{0}C. It is found that the desorption is complete in a few minutes. When the first sample is taken after one min 90-100 % of the enzyme activity is already present in the supernatant. This fast desorption rate is also found for powders which were loaded by prolonged incubation (12 h) in the adsorption reaction. When a diffusion coefficient of 1.0×10^{-11} m^2/s is assumed for the diffusion of α-amylase in the matrix, a Fourier analysis shows that for the fully loaded particles diffusion is completed in a time in the order of 1-10 min. This value is larger than the experimentally found values. The fast desorption and partial adsorption at short reaction times indicate that the adsorption has only taken place at the particle outer surface.

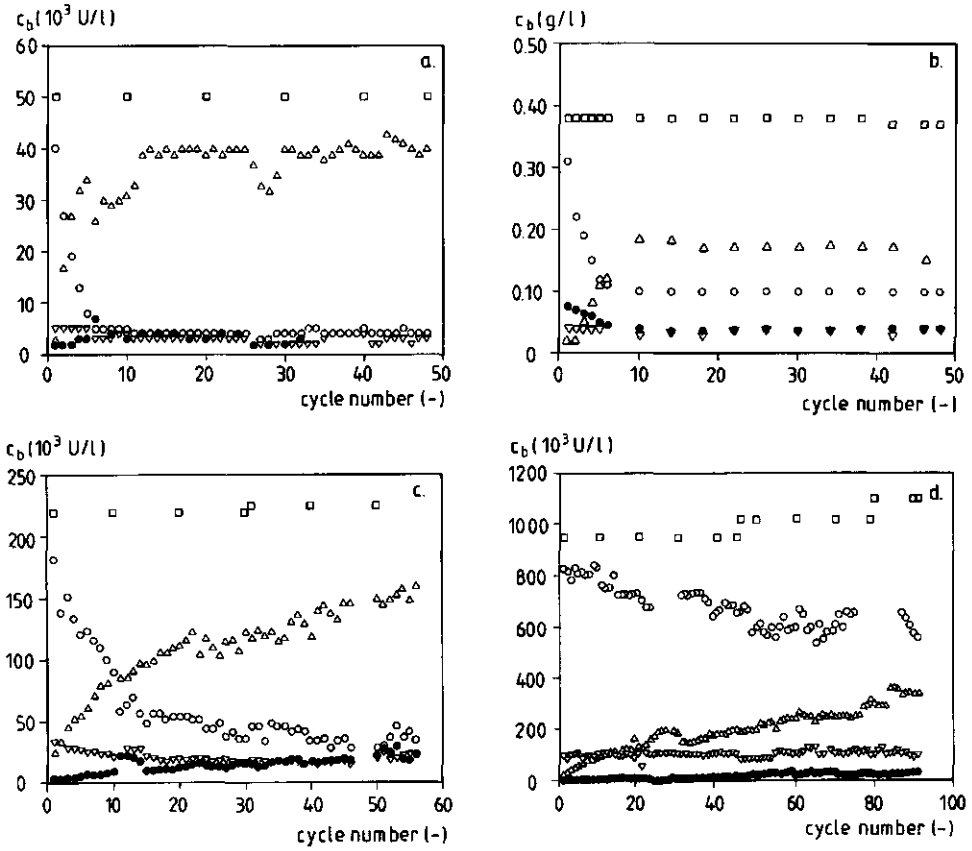

Fig. 5.3 - Repeated adsorption of alpha-amylase on crosslinked starch: enzyme (a,c,d) and protein (b) concentrations of various fractions. □ *: starting solution;* ○ *: solution after adsorption;* ● *: wash;* △ *: desorption;* ▽ *: regeneration; (a,b):* $c_{b,0} = 50 \times 10^3$ *U/l. Matrix M1; (c):* $c_{b,0} = 225 \times 10^3$ *U/l. Matrix M2; (d):* $c_{b,0} = 950\text{-}1100 \times 10^3$ *U/l. Matrix M3. Buffer: 100 mM sodium acetate pH 6.0; T = 4 ^{0}C.*

Repeated adsorption and desorption reactions

Development of adsorption characteristics

Repeated adsorption and desorption reactions were carried out with the matrices using different enzyme concentrations in the adsorption experiment. The powders were used extensively for fifty to hundred runs. Figure 5.3 shows the development of the adsorption process for three starting concentrations of enzyme (matrices M1,M2,M3 (Table 5.3)). During the experiment the adsorption levels increase for all three enzyme concentrations (see Fig. 5.3(a/c/d)). It shows that the exposure of the matrix to the enzyme results in increased binding of the adsorbate. In the end this results in adsorption levels which are a factor of 1.5-5 higher than the values obtained for the matrix at the start of the experiment.

Table 5.3 - Repeated adsorption and desorption of ∝-amylase to crosslinked starch powder. Adsorption characteristics of crosslinked starch.

enzyme	adsorbent						
	number	concentration		breakdown	loading		average particle diameter
		start	end		Theoretical[a]	Experimental[b]	
(U/l)		(g/l)		(%)	(U/g)		(10^{-3} m)
-							0.43±0.16
50,000	M1	24	15.2	37	1,900	3,000	0.35±0.18
225,000	M2	33.3	13.8	59	5,000	14,000	0.25±0.1
950,000	M3	27.7	12.9	53	6,000	29,000	0.35±0.19

[a]*Obtained from adsorption isotherm;* [b]*Calculated from experimental results.*

Figure 5.4 shows the measured adsorption isotherm for the matrices together with the adsorption isotherm of the crosslinked starch starting product. Although there is a lot of scatter in the adsorption data it is clear that the adsorption characteristics of the matrices are comparable for the three matrices and that the capacity is considerably different from that of the same matrix in the first adsorption cycle. The adsorption capacity of the products estimated from Fig. 5.4 is between 9.4 × 10^7 U/kg and 16.6 × 10^7 U/kg, being 15-25 times larger than at the start of the experiment (see Table 5.1). Using these capacity values for an estimation of K_S a value of 145 × 10^3 -250 × 10^3 U/l is obtained, while K_S is 13.3 × 10^3 U/l at the start of the experiment. These values show that as a result of the process the affinity of the enzyme for the matrix is decreased and that the capacity of the support is enlarged compared to the starting material (Table 5.1).

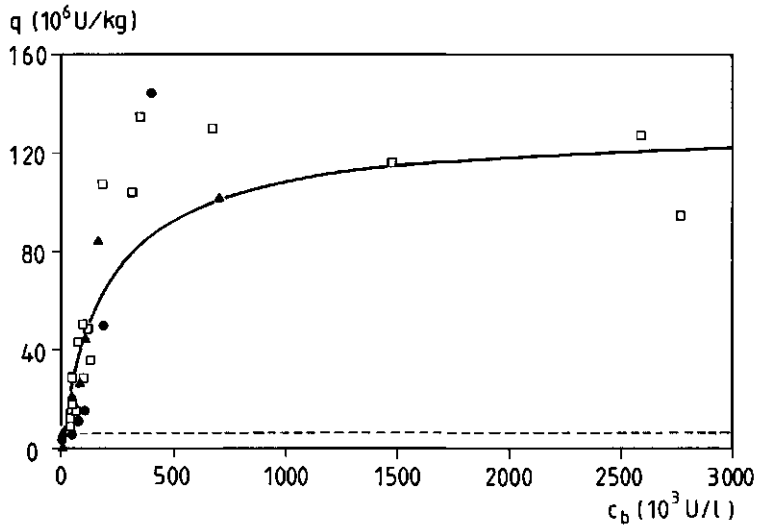

Fig. 5.4 - Adsorption isotherms of alpha-amylase on crosslinked starch.----: crosslinked starch (starting product, Fig. 5.1(a));
□ . ▲ . ● *: equilibrium data matrices M1, M2 and M3 respectively;*
———— *: adsorption isotherm, K_S = 200 × 10^3 U/l, q_m = 13.0 × 10^7 U/kg; Buffer: 100 mM sodium acetate pH 6.0; T = 4 °C.*

Due to these changes in the binding characteristics the performance of these matrices still improves after 60-90 cycles as the levels of adsorption and desorption still increase (see Fig. 5.3(c,d)).

The increase in the adsorption level can be explained by assuming that the matrix becomes more porous. This also agrees with the conclusion that in the first adsorption experiments the enzyme is adsorbed only in the outer region of the particle. The fact that the matrix becomes more porous due to the interaction with enzyme was confirmed by comparing the gel filtration properties by measuring the distribution factor K_d ($K_d = (V_e-V_0)/V_p$) of a crosslinked starch before the first adsorption cycle and an adsorbent which had been exposed to the enzyme as described in "Material and Methods". Bacitracine (1440 D), lysozyme (14.4 kD) and chymotrypsinogen (25 kD) were more retarded on an enzyme exposed column consisting of crosslinked starch (K_d = 0.64, 0.34 and 0.15 respectively) than on a column of freshly prepared crosslinked starch (K_d = 0.42, 0.17 and 0.05 respectively). Ovalbumin (43 kD), BSA (68 kD) and ferritin (450 kD) were not retarded by either of the materials under the elution conditions chosen. It is shown that the fractionation range of crosslinked starch increases when the matrix is exposed to α-amylase showing that as a result of the incubation with the enzyme the matrix becomes better accessible for larger molecules.

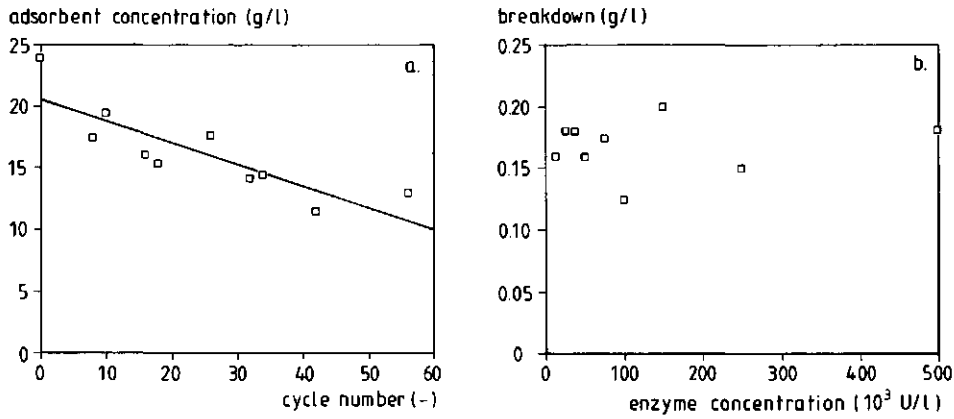

Fig. 5.5 - Biodegradation of adsorbent during repeated adsorption/ desorption experiment. (a) Concentration of adsorbent; Initial concentration: 20 g/l. (b) Biodegradation of crosslinked starch as function of enzyme concentration; Adsorbent concentration: 20 g/l.

For the experiment shown in Fig. 5.3(a) the actual breakdown of the matrix has been observed (Fig. 5.5(a)). Although the data points are scattered it appears that the breakdown of the adsorbent does not follow the same trend as the changes in adsorption characteristics. In the cycles 20-90 where no

increase of adsorption capacity is found, yet the matrix degradation proceeds. Furthermore breakdown of the matrix appears to be independent of the enzyme concentration in solution (Fig. 5.5(b)). For two of the three experiments an average breakdown of 0.16 g/l per cycle was found, for the third experiment 0.34 g/l was found.

It can be explained that the capacity of the three matrices, which were exposed under different conditions (enzyme concentration and number of cycles), is approximately the same. In the initial part of the experiment binding sites become available for the enzyme resulting in increasing adsorption levels. In the case that the whole particle is attainable for the enzyme, further breakdown does not result in a net increase in attainable binding sites.

Mass transfer

The adsorption process reaches a more or less constant pattern in the last cycles of each experiment which allows the evaluation of the adsorption kinetics for these matrices. Figure 5.6 shows the adsorption data for the three matrices at two starting concentrations of enzyme. Compared to the starting product (crosslinked starch) the kinetics are drastically different (see for example Fig. 5.2(a)).

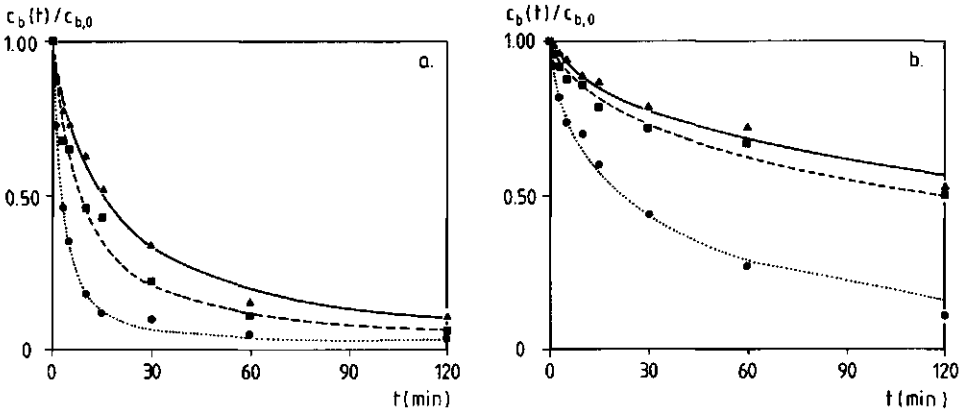

Fig. 5.6 - Modelling of the adsorption of alpha-amylase on crosslinked starch after 100 cycles with the extended pore diffusion model. Parameters as in Tables 5.4 and 5.5. (a) $c_{b,0} = 205 \times 10^3$ U/l. ▲ : M1; ● : M2; ■ : M3; (b) $c_{b,0} = 850 \times 10^3$ U/l. ▲ : M1; ● : M2; ■ : M3. Adsorbent concentration: 23.1 g/l. T = 4 °C.

It appears that the adsorption levels found after 120 min of adsorption are not described with the value range of K_S (145 × 10^3 U/l - 250 × 10^3 U/l) and q_m (9.4 × 10^7 U/kg - 16.6 × 10^7 U/kg) found from the adsorption isotherm of matrices M1, M2 and M3. After 120 min with $c_{b,0}$ = 205 × 10^3 U/l the concentration in the liquid phase is 8.0 × 10^3 U/l whereas the isotherm predicts an equilibrium liquid concentration of 13.2 × 10^3 U/l. This can be explained by the fact that after a fast initial adsorption biodegradation starts and oligosaccharides go into solution. This results eventually in a partial release of some of the bound enzyme from the adsorbent. The isotherms were determined at equilibrium conditions, i.e. including the oligosaccharides in solution. When the adsorption level is monitored over a longer period (48 h) an increase of the enzyme concentration in the liquid phase is indeed observed and the values predicted by the isotherm are found.

Table 5.4 - Reaction parameters for amylase/crosslinked starch system.

adsorbent	**value**
ρ_p	: 1.05 × 10^3 g/l
q_m	: 130 × 10^6 U/kg
R	: 0.125-0.175 × 10^{-3} m[a]
equilibrium and rate parameters	
k_l	: 10^{-6} - 10^{-5} m/s[b]
K_S	: 13.3 × 10^3 U/l
k_i	: 10/s
k_d	: 1.33 × 10^5 U/l.s[c]
D_S	: 10^{-13} - 10^{-12} m^2/s[d]
dimensionless parameters[e]	
Bi	: 130-2900
Φ	: 125-715
λ[f]	: 0.1-4.2
ν	: 125-175

[a]: see Table 3; [b]: obtained from ref. [24]; [c] : calculated with values of K_S and k_i; [d]: obtained from ref [28]; [e]: range for the experiments (Table 5.5) calculated with parameters from Tables 5.4 and 5.5; [f]: value for D_p: 6.0 × 10^{-11} m^2/s. from literature correlations.

The adsorption data were modelled with an extended pore diffusion model as previously used [21]. For the calculations the parameters given in Table 5.4 were used. With the values of K_S of 145 × 10^3 U/l - 250 × 10^3 U/l no good simulation of the adsorption process could be found. As discussed above the adsorption process is complex in the initial 120 min and it is not described by the adsorption equilibrium value of K_S for the matrix. Therefore the value of K_S (13.3 × 10^3 U/l) found for the crosslinked starch powder was used as the parameter for calculation (see Table 5.1). The value for k_i was calculated by fitting the model to the experimental data from an experiment as described under **"Adsorption kinetics"** in which external mass transfer and kinetics of complex formation are rate limiting (initial enzyme concentration: 40 × 10^3 U/l, other conditions as in Fig. 5.6). The value of k_l, as obtained from literature data [21,24], was set on 1 × 10^{-5} m/s. This yields a calculated value of 10/s for k_i. When these values are introduced in the dimensionless numbers describing the adsorption process (eqns. 1-4 and Table 5.4) it follows that pore diffusion will govern the overall adsorption reaction.

Table 5.5 - Simulation of adsorption kinetics with the extended pore diffusion model.

$c_{b,0}$ (U/l)	matrix	D_{eff} (calculated) (10^{-11} m^2/s)
205 × 10^3	M1	0.3
	M2	1.0
	M3	0.5
850 × 10^3	M1	0.06
	M2	0.25
	M3	0.09

The model predictions for the adsorption are given as solid lines in Fig. 5.6. In Table 5.5 the calculated diffusion coefficients are collected. Generally the adsorption is described quite well by the set of parameters used. The

values for the diffusion coefficient are smaller than the values for the diffusion of α-amylase in water being approximately 6.0×10^{-11} m^2/s, obtained from literature correlations [26]. Furthermore, a strong dependency of the calculated diffusion velocity on the bulk enzyme liquid concentration is observed. The dependency of the diffusion velocity on the concentration ($c_{b,0}$) is reported in literature [23,27,28] and may be explained by the fact that surface diffusion is of importance at the concentration of 205×10^3 U/l. Another explanation is that at liquid concentrations of 850×10^3 U/l due to the high loading capacity of the support (0.6 g protein/g adsorbent) diffusion becomes restricted [30].

Matrices M1 and M3 behave similarly in the adsorption of enzyme. Their porosity appears to be comparable. Matrix M2 is more porous resulting in higher values for D_{eff}. This matrix is the most extensively degraded as is clear from both the average particle diameter and the total amount of breakdown products determined in the repeated experiments (see Table 5.3). This agrees with the assumption that increased biodegradation results in a more porous adsorbent.

Table 5.6 - Repeated adsorption and desorption of α-amylase on crosslinked starch powder: Enzyme activity (U/l), protein content (g/l) and specific activity (U/g) of subfractions.

Adsorbent	M1			M2			M3		
	(10^3 U/l)	(g/l)	(10^3 U/g)	(10^3 U/l)	(g/l)	(10^3 U/g)	(10^3 U/l)	(g/l)	(10^3 U/g)
Enzyme solution	50	0.38	132	225	1.7	133	1020	7.5	136
Adsorption (2)	5	0.1	50	35	0.5	70	600	5.4	103
Wash step (3)	2	0.04	50	17	0.25	70	100	0.3	110
Desorption (4)	40	0.17	235	140	0.6	230	340	1.5	225
Regeneration (5)	2	0.04	50	18	0.25	70	30	0.3	100
Total(2+3+4+5)	49	0.35		210	1.6		1070	7.5	

Recovery and specificity

Recovery of enzyme activity and protein is very good in this repeated process and is between 92-100% in all the experiments. Data for the last ten runs of the experiments are collected in Table 5.6. A purification factor of approximately 1.7 is achieved which is in agreement with the results obtained earlier [18,19]. The desorption fraction (see Table 5.6) is electrophoretically pure [18]. Independent of the bulk liquid concentration, there is no change in specificity in the concentration range examined.

The adsorption process is characterized by a shifting equilibrium line for adsorption. Figure 5.7 shows the adsorption isotherm of Fig. 5.4 with the shifting equilibrium lines for one concentration of $c_{b,0}$ (1000×10^3 U/l) at proceeding matrix biodegradation. When a linear relationship between the cyclenumber and the biodegradation and a capacity $q_m = 13.7 \times 10^7$ U/kg are assumed, then the adsorbent can be used for at least 140 cycles at $c_{b,0} = 1000 \times 10^3$ U/l under the experimental conditions assuming mass transfer equilibrium to be reached.

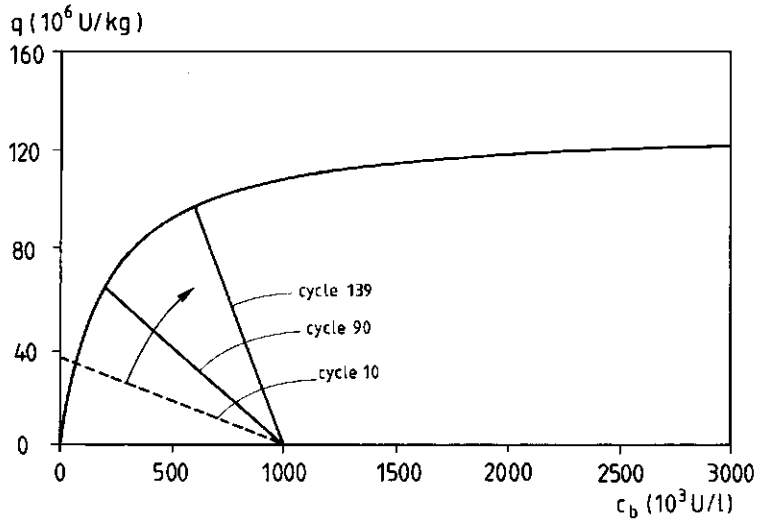

Fig. 5.7 - Equilibrium lines for adsorption of α-amylase on crosslinked starch. $c_{b,0} = 1000 \times 10^3$ U/l; Matrix M3.

The equilibrium, characterized by the corresponding equilibrium line, is shifting as a result of the adsorbent degradation. This results in increased

loading values (q) for the matrix in every run (see Fig. 5.7). However the total amount of enzyme bound in each following run will decrease. With this process approximately 9.5 kg pure α-amylase will be obtained with 1 kg adsorbent. When breakdown is independent of the enzyme concentration over a large range and the whole capacity of the support is used in every run approximately 40 kg of enzyme can be finally obtained with 1 kg adsorbent. However in that case the cycle times have to be considerably longer than for the case described above.

5.5 CONCLUSIONS

In previous papers the development of an affinity adsorbent for bacterial α-amylases was reported [15,18,19]. The main problem in the application of these adsorbents in the isolation of the enzyme was the very slow adsorption kinetics of this system which made it only suitable as a low capacity affinity system for analytical purposes. However, when the equilibrium conditions of the system were studied it was found that the capacity of the adsorbent for the protein was much larger than initially expected from the kinetic experiments.

This effect is explained by two phenomena which affect the binding of α-amylase to this kind of products. Firstly, the prepared crosslinked products consist of relatively large particles in which most of the binding sites are not directly available for the enzyme. When the average particle size of the crosslinked starch was decreased by a grinding step a product was obtained with considerably better adsorption characteristics for the enzyme. This can be contributed to the larger surface area which has become available for binding. However, even then the matrix appears to be accessible to a limited extent only. Further improvement can be achieved by enzyme-adsorbent interaction. As a result of this interaction the matrix gets better accessible for the enzyme. This can be explained by the fact that enzymic activity on the matrix will result in a more porous structure which allows an increased penetration of the protein into the adsorbent resulting in improved adsorption characteristics. This increase in porosity was confirmed by comparing two adsorbents with respect to their gel filtration properties, resulting in a larger fractionation range for the adsorbent that was incubated with the enzyme. Furthermore, the amount of binding sites becomes 15-30 fold enhanced in the course of the enzymatic modification process.

As shown the adsorption of α-amylase to the adsorbent is accompanied

by some biodegradation. The kinetics of the complex formation and the equilibrium values finally reached are determined by the competition of the enzyme for the adsorbent and the soluble oligomers. Therefore in the initial stage where biodegradation has not fully evolved, the adsorption process is characterized by a lower value for K_s than the one obtained from the equilibrium data. Although the matrix is degraded during the process, the loss of material is fully made up for by an improved mass transfer, resulting in still increasing adsorption levels after 90 cycles.

Acknowledgement

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NOMENCLATURE

Bi : Biot number (-)
c : local concentration of enzyme in the pore liquid phase (U/l)
c_b : bulk liquid concentration (U/l)
$c_{b,0}$: liquid concentration at t = 0 (U/l)
$c_b(t)$: batch liquid concentration at time t (U/l)
D_{eff} : effective pore diffusion coefficient of protein in particle (m^2/s)
D_p : diffusion coefficient of protein in particle pore (m^2/s)
D_s : diffusion coefficient of protein due to surface diffusion (m^2/s)
D_w : diffusion coefficient of protein in liquid phase (m^2/s)
k_i : rate constant local adsorption (l/s)
k_d : rate constant local desorption (U/l.s)
k_l : liquid film transport coefficient (m/s)
K_d : distribution factor (-)
K_s : Langmuir constant (U/l)
m_p : mass of matrix (g)
M : molecular weight (D)
P_e : enzyme partition coefficient (-)
q : concentration of the enzyme on the adsorbent (U/kg)
q_m : capacity of the adsorbent (U/kg)
R : radius of particle (m)
t : time (s)
v_i : local adsorption velocity (U/l.s)
V_e : elution volume (l)
V_l : volume liquid phase (l)

V_0 : void volume (l)

V_p : pore volume (l)

Greek symbols

λ : parameter defined in eqn. 3 (-)

ν : parameter defined in eqn. 4 (-)

ρ_p : density of wet support (g/l)

Φ : Thiele modulus (-)

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CHAPTER 6

ALGINATE-STARCH COPOLYMERS AND IMMOBILIZED STARCH AS AFFINITY ADSORBENTS FOR α-AMYLASE

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SUMMARY

Novel adsorbents for the isolation of α-amylase are described. The first adsorbent is a copolymer of alginate and starch. Water soluble starch was converted into a bead form by calcium chloride complexation of an alginate/starch mixture. The starch was made insoluble by a crosslinking reaction with epichlorohydrin. Another type of adsorbent is obtained by immobilization of crosslinked starch in an alginate matrix.

The preparation of the adsorbents was optimized with respect to adsorption capacity and enzymic breakdown by α-amylase. The two adsorbents prove to be useful in the purification of amylases from diverse sources. With respect to the equilibrium conditions they have comparable properties. Mass transfer is more restricted in the first adsorbent, probably due to increased diffusion resistance in the copolymer matrix.

In addition these adsorbents may be useful for the direct recovery of the enzyme from unclarified fermentation media or cell homogenates.

Submitted for publication.

6.1 INTRODUCTION

Adsorption of polysaccharide degrading enzymes on their substrates is a procedure to isolate these enzymes from various sources [1]. To obtain a rigid matrix, suitable for packed bed chromatography, mostly water soluble polysaccharides are made insoluble by a crosslinking reaction, thus yielding low cost, easily prepared matrices. The most frequently used crosslinking reagent in these reactions is epichlorohydrin. Purification procedures for several polysaccharide degrading enzymes like α-amylase [2,3], endo-polygalacturonase [4,5], cellulase [3] and pectate lyase [6] on their crosslinked substrates were described. Recently stable matrices for endo-polygalacturonase [5], β-amylase [7] and α-amylase [8] were developed. Alginate, a substrate analogue of pectate, is able to bind endo-polygalacturonase while no breakdown of the matrix is observed [9,10]. Crosslinked starch or starch derivatives are useful affinity adsorbents for the isolation of bacterial α-amylases [8,11,12]. For example a procedure was developed by crosslinking potato starch with epichlorohydrin in water-ethanol mixtures under alkaline conditions. A suitable crosslinked starch was synthesized by optimizing the important reaction parameters (epichlorohydrin concentration, ethanol-water ratio, sodium chloride-epichlorohydrin ratio, reaction time and temperature) in the crosslinking reaction [11]. The crosslinked starch powder can be used to isolate the enzyme from complex mixtures. Almost no enzymatic breakdown occurs while the binding capacity is approximately 13 U /mg adsorbent [11,13].

To apply an affinity adsorbent directly in unclarified broths or homogenates the matrix has to be transformed into particle form. The direct recovery of products from fermentation media is interesting because it can replace a number of process steps now in use in the purification of proteins from process media (e.g. filtration, centrifugation and precipitation). Application of particles enables the use of a fluidized bed reactor for the isolation of proteins in a one step procedure. In this way common problems in packed bed chromatography, like clogging and fouling, are avoided. Alginate matrices are widely used to immobilize enzymes and cells for biocatalysis [14]. The use of alginate as a material to prepare adsorbents was only recently introduced [10,15-17]. Isolation of endo-polygalacturonase from a commercial enzyme sample was described using alginate beads as the affinity sorbent [10]. These alginate beads can also be used very effectively to isolate endo-polygalacturonase from complex mixtures [10]. Isolation of β-lactamase from a Bacillus homogenate was described using alginate entrapped DEAE-trisacryl [15].

In this paper beaded affinity adsorbents are described in which the gelling properties of the alginate are combined with the adsorption properties of the crosslinked starch. The beads can be stabilized with respect to enzymatic breakdown whereas the capacity of the supports appears to be very well comparable with the adsorbents described previously [13,18].

6.2 MATERIAL AND METHODS

Drum dried starch powder from potato (Paselli WA4) is a product of AVEBE, Veendam, The Netherlands. Sodium alginate (Manucol DM) is a product of Alginate Industries (London, UK). Maxamyl (a heat stable α-amylase from B. licheniformis) is a product of Gist-brocades N.V. (Delft, The Netherlands).

Determination of enzyme activity and protein content

α-amylase activity is determined by an automated neocuproin test using an autoanalyzer (Skalar, Breda, The Netherlands) as described previously [9]. Pullulanase activity is determined as described previously [13]. Protein content is determined by using the method according to Lowry [19]. In some cases a precipitation step is included in the protein assay.

Preparation of alginate/starch copolymers

a. Preparation of beads

6 g of drum dried starch powder is added to 100 ml of 50 mM sodium acetate pH 6.0 and solubilized with an ultraturrax stirrer. Subsequently 1 g sodium alginate is added and solubilized. This viscous solution is deaerated at room temperature for approximately 12 h. Beads are prepared by dropping the solution under pressure through a glass capillary in a 200 mM calcium chloride solution in 50 mM sodium acetate pH 6.0. The gelation reaction is carried out for 1 h. Then the beads are washed and stored in 50 mM sodium acetate with 10 mM calcium chloride.

b. Crosslinking of beads

The beads are dehydrated in 1 h by replacing the buffer with a series of ethanol solutions (10-96%). The beads are retained in 96% ethanol and heated to the desired reaction temperature in a shaking incubator (90 min). Epichlorohydrin is added in a desired concentration, the crosslinking ratio (CR)

being the ratio between the moles of epichlorohydrin and the moles of glucose moieties at the start of the reaction. After 20 min 10.5 M sodium hydroxide is added in an equimolar concentration with respect to the epichlorohydrin. The reaction is stopped by the addition of an excess of 7% (v/v) acetic acid. The crosslinked beads are hydrated by replacement of the solution by a series of ethanol/water mixtures (20-100% (v/v) water). The beads are stored in a 50 mM sodium acetate buffer pH 6.0 with 10 mM calcium chloride.

Preparation of beads composed of starch immobilized in alginate

Crosslinked starch is prepared and ground before immobilization in a Retsch grinding device as described previously [18]. Alginate is dissolved in distilled water in the desired concentration (0.5 - 2.5% w/v). The alginate is solubilized by using an ultraturrax mixer (5 min). Ground starch powder was added in different concentrations (5 - 20% w/v). Beads are prepared by dropping these solutions in a 0.5 M calcium chloride solution with continuous stirring. The bead diameter can be manipulated by varying the needle opening and by using an air stream to blow off the forming drops. The beads are stirred for 24 h in 0.5 M calcium chloride. Subsequently they are equilibrated against and stored in a 0.1 M calcium chloride solution.

Determination of the bead diameter

The bead diameter is determined by using a binocular microscope (Zeiss-Ikon) with a measuring device. The diameter used in the calculations was the average value obtained by measuring the diameter of 20-30 beads.

Dry weight determination

In a calibrated tube 5, 12.5, 25, 50, 100 and 150 g of ground crosslinked starch is added to 500 ml 0.5% (w/v) alginate in distilled water. After mixing the total volume of the solution (and thus the concentration of starch) is determined. Subsequently beads are prepared as described above. The amount of beads is determined by measuring the volume occupied by the particles in a calibrated tube. This amount of beads is dried in an oven for 24 h (90 ^{0}C). The weight of the residue is determined on an analytical balance and the concentration of starch in the particles is calculated. For other alginate concentrations in the beads the same procedure is followed as described above.

Starch content of the beads

a. Hydrolysis

The beads are sieved off and dried with a tissue. Subsequently approximately 300 mg of beads are placed in a kimax tube. The tube is placed on ice and 0.9 ml 72% (w/w) H_2SO_4 is added. The tube is incubated for 1 h at 30 ^{0}C in a water bath. After the hydrolysis step 9.9 ml distilled water is added. The reaction mixture is heated for 3h at 100 ^{0}C in an oil bath.

b. Determination of total sugar content

Determination of total sugar content is performed according to Dubois [20]. The samples are measured at 490 nm. The reference is a standard of starch (Paselli WA4, AVEBE, The Netherlands).

c. MHDP test for uronic acid content

To 0.3 ml of a 1:10 diluted sample obtained after hydrolysis 3.6 ml 0.125 M sodium tetraborate is added. The sample is heated at 100 ^{0}C (5 min). Subsequently 60 μl MHDP (0.15% (w/v) in sodium hydroxide (0.5% (w/v)) is added to the reaction mixture. The absorption is measured at 520 nm. As the standard alginate is used.

Determination biodegradation

The beads are assayed for biodegradability by α-amylase by incubating them for 24 h in adsorption buffer at 4 ^{0}C (100 mM sodium acetate pH 6.0 with 10 mM calcium chloride) or in desorption buffer (100 mM sodium acetate pH 6.0 with 10 mM calcium chloride and 20 % (v/v) glycerol) at 40 ^{0}C. To this end 1.5 g of beads are suspended in 15 ml of buffer with an enzyme concentration of 50 U/ml. At chosen time intervals samples (200 μl) are taken. These samples are further incubated in 790 μl 100 mM sodium acetate pH 5.0 with the addition of 10 μl amyloglucosidase (Sigma, St. Louis, USA) for 16 h at 30 ^{0}C to hydrolyse the (poly)saccharides present. The samples are assayed for reducing end groups by means of an automated neocuproin test using an autoanalyzer (Skalar).

Adsorption equilibria

Adsorption isotherms were determined at 4 ^{0}C by incubating 1.5 g of copolymer beads and 0.5 g of immobilized starch in 15 and 20 ml adsorption buffer respectively to which varying enzyme concentrations (0-500 U/ml) are added. The amount of adsorbed enzyme was found by measuring the decrease

of enzyme activity in the liquid phase at equilibrium after 72 h [21].

Adsorption and desorption kinetics

For the adsorption kinetics, typically 40-200 mg/ml of adsorbent is suspended in enzyme solution (5-250 U/ml) and incubated in a rotating incubator (Labinco, 40 rpm) or on a magnetic stirrer at 4 ^{0}C. Sampling is done by taking 20-200 μl of the liquid fraction at chosen time intervals. The sample is further diluted for the enzyme activity assay.

Desorption kinetics are measured by incubating the enzyme-loaded adsorbent (40-200 mg/ml) in desorption buffer at 70 ^{0}C. Sampling is done as described above. The change in the volume of the liquid fraction as a result of sampling was always less than 10% in these experiments. Desorption can also be accomplished by incubating the beads with a diluted glucose syrup (20-50 mg/ml). Sampling is performed as described above.

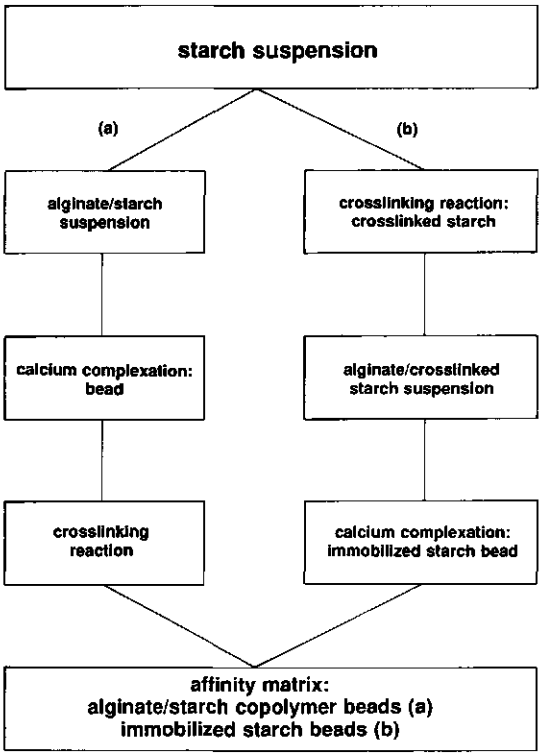

Fig. 6.1 - Preparation of beaded affinity adsorbents for α*-amylase.*

6.3 RESULTS

To obtain beaded adsorbents based on starch and alginate, two different strategies have been followed (Fig. 6.1). Crosslinked starch is either directly immobilized in alginate (path b) or a mixture of alginate and soluble starch is crosslinked to form a stable copolymer after Ca^{2+}-complexation of the mixture (path a).

Table 6.1 - Influence of the reaction parameters on the breakdown levels of alginate/starch copolymer beads.

CR	matrix	reaction temperature (^{0} C)	reaction time (h)	breakdown level (24 h) adsorption medium (4 ^{0}C) (%)	desorption medium (40 ^{0}C) (%)
5	A	53	8	34	27
	B	60	4	28	27
	C	60	6	22	27
	D	60	8	4-7	8-11
6	E	40	8	55	56
	F	53	8	19	14
	G	60	4	9	14
	H	60	6	3-4	7-8
	I	60	8	2-3	4-5
7	J	60	8	0.7	2.9
8	K	60	8	0.7	1.4
10	L	60	8	0.4	1.8

Synthesis of alginate/starch copolymers

Matrices were prepared from solutions with 1 g sodium alginate and 6 g starch per 100 ml of water. 1% (w/v) alginate is necessary to obtain a rigid bead. When using amounts of starch larger than 6% w/v the solution becomes

too viscous to be used in the bead preparation procedure. The copolymer bead contains 89.9 mg starch/g bead as determined by the Dubois test.

Influence of the reaction parameters on the stability of alginate/starch copolymers

Previously it was shown that suitable adsorbents can be obtained in different ways by altering the main parameters (i.e. the absolute epichlorohydrin concentration, the ethanol/water ratio, the sodium hydroxide/water ratio and the reaction time and temperature) [11].

Beads are prepared at different reaction temperatures with two crosslinking ratios (CR = 5 and 6) and one reaction time (8 h). In Table 6.1 the breakdown levels of the matrices are summarized. The breakdown level is strongly dependent on the reaction temperature applied. With increasing temperature more stable matrices are obtained. At a reaction temperature of 60 ^{0}C products are obtained with breakdown levels of about 5 % (after 24 h). These results are confirmed in experiments where the reaction time is varied (Table 6.1). The data show that prolonged reaction times (up to 8 h) are needed to obtain matrices that are stable enough for use in adsorption experiments (biodegradability smaller than 5% in 24 h).

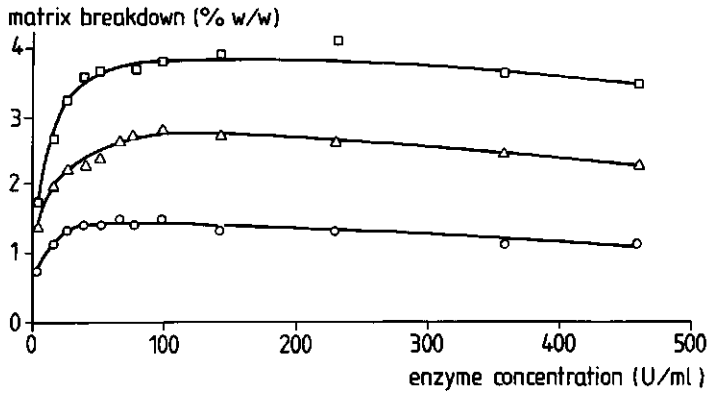

Fig. 6.2 - Biodegradation of alginate/starch copolymer beads by α-amylase. Adsorbent concentration: 20 g/l; Enzyme concentration: 50 U/ml; T = 4 ^{0}C; Buffer: 100 mM sodium acetate with 10 mM calcium chloride; Incubation time: 48 h. □ *: Matrix H;* △ *: Matrix I;* ○ *: Matrix K.*

At CR = 5 matrices can be obtained that are equally stable as the ones obtained at a CR of 6 (Table 6.1), however, with longer reaction times. In Table 6.1 also breakdown levels are given for beads obtained by performing the reaction for 8 h at 60 ^{0}C with different CR values. The highest stability is obtained at the highest crosslinking levels (CR 8 and 10). The breakdown by α-amylase is reduced to less than 2% in 24 h at 4 ^{0}C with these matrices. It appears that above 50 U/ml the amount of biodegradation is not increasing with an increasing concentration of enzyme in solution (Fig. 6.2).

Breakdown levels are reduced approximately a factor 10 by increasing the glycerol concentration from 0 to 50% (v/v) (Table 6.2). Previously it was shown that a decreasing V_{max} of the enzyme and a decreasing interaction of the adsorbate with the adsorbent are responsible for this phenomenon [13].

Table 6.2 - Influence of the glycerol concentration on the breakdown levels of alginate/starch copolymer beads at 40 ^{0}C (matrix I).

glycerol concentration (v/v)	breakdown (%)
0	5.8
10	4.0
20	2.9
30	1.7
40	0.9
50	0.7

Properties of the adsorbents

The starch content of the beads is determined for the copolymers as well as for the starch immobilized in alginate. The volume and the dry weight of the beads are determined. Upon immobilization of crosslinked starch in alginate by Ca^{2+}-complexation shrinking occurs which is dependent on the amount of starch in the suspension (Fig. 6.3). The reduction of volume ($V_{bead}/V_{suspension}$) ranges from a factor 1.3 (20-26% starch (w/v)) up to 2.4 (1% starch (w/v)). The density of the (20-26% starch (w/v)) beads is 1050±50

kg/m^3. In Table 6.3 the sugar (starch) contents of various beads are collected. The results confirm the values obtained from the complexation experiments. The starch content of the copolymers does not change as a result of the crosslinking reaction because the beads do not change in size. The starch content of the beads composed of starch immobilized in alginate is larger than that of the suspension.

Table 6.3 - Starch content of beads composed of starch immobilized in alginate and of alginate/starch copolymer beads.

bead type		starch content beads (mg starch/g bead)	
		total sugar determination	dry weight determination
starch immobilized in alginate			
alginate (% w/v)	starch (% w/v)		
0.5	5	100	80
0.5	10	192	191
0.5	15	230	192
0.5	20	243	238
1.0	5	121	129
1.0	10	165	167
1.0	15	202	198
1.0	20	218	209
alginate/starch copolymer (matrices H,I and K)		90	n.d.[a]

[a] *n.d.= not determined.*

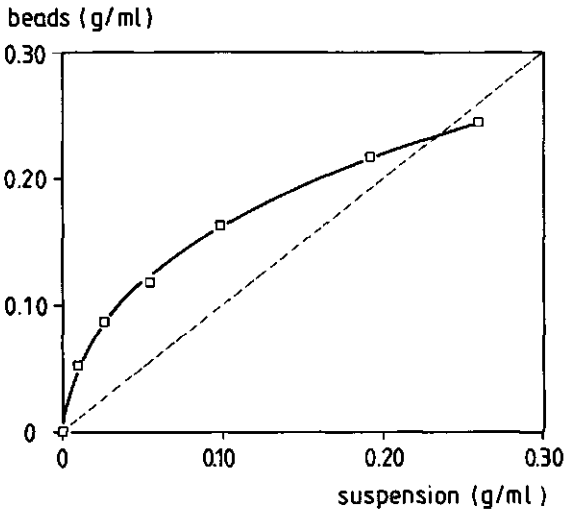

Fig. 6.3 - Starch content of beads composed of starch immobilized in alginate.

Adsorption equilibria

For the copolymers the adsorption isotherms were determined for matrices where the breakdown levels are below 5% (matrices H (CR 6), I (CR 6) and K (CR 8)). In Table 6.4 the Langmuir constants and the capacities of the supports for the enzyme are collected. The capacity of the adsorbent with CR 8 is lower than of the adsorbents prepared at CR 6. This can be explained by the higher degree of crosslinking in these beads which results in a lower number of binding sites per g of starch. The capacity (q_m) of the copolymer adsorbents with CR 6 (matrices H and I) is up to 3 times larger than the values for crosslinked starch [13], for the copolymer with CR 8 (matrix K) q_m is about the same. For the beads composed of starch immobilized in alginate the capacities (q_m) are approximately the same as the capacity of the crosslinked starch. The adsorption of α-amylase to the alginate in the beads is negligible (Table 6.4).

Adsorption kinetics

In Fig. 6.4 the adsorption of α-amylase (50 U/ml, resp. 100 U/ml) to

immobilized starch beads (10% starch (w/v)/1% alginate (w/v)) is shown. The activity and protein adsorption patterns deviate. After 60 min a specific activity of about 230 U/mg is calculated for the adsorbed fraction of enzyme, a value comparable with previous results with crosslinked starch [13,18].

Table 6.4 - Adsorption isotherms - Langmuir constants and capacities of supports.

support	K_S/q_m	q_m		K_S
	(mg/ml)	(U/g bead)	(U/mg starch)	(U/ml)
crosslinked starch	3.6±0.3	-	9.2±1.1	33±6
alginate/starch (% w/v)				
1.0/ 0.0		0[a]	0[a]	
0.5/ 5.0		n.d.	n.d.	
0.5/10.0	2.4±0.3	2260	12±3	29±5
0.5/15.0	2.7±0.3	2910	13±4	35±6
0.5/20.0	2.4±0.4	4600	19±6	46±8
1.0/ 5.0		n.d.	n.d.	
1.0/10.0	2.6±0.2	1670	10±2	26±4
1.0/15.0	2.0±0.3	2020	10±2	20±4
1.0/20.0	1.9±0.3	2030	9.5±2	18±4
CR 6, H	3.2±0.3	2520	28±6	90±20
I	2.3±0.3	2840	32±6	72±12
CR 8, K	5.6±0.5	650	7±2	40±6

[a] *No adsorption was detected after incubation of 0.5 g 1% (w/v) alginate beads in 20 ml Maxamyl (12.5 U/ml).*

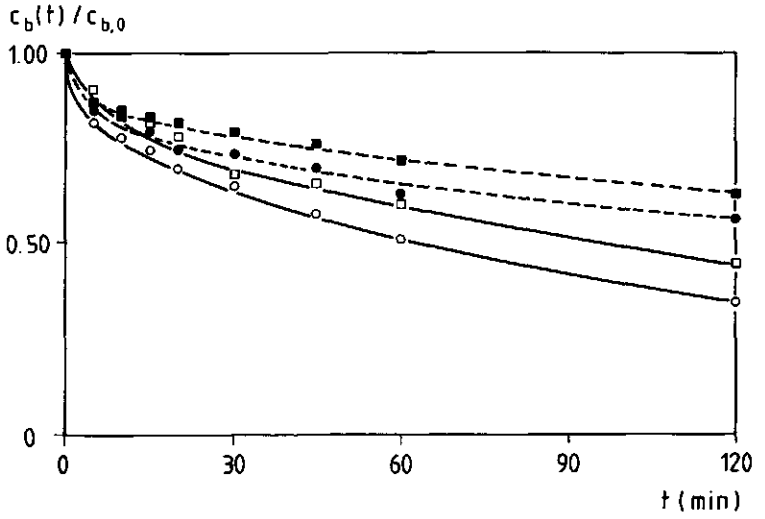

Fig. 6.4 - Adsorption of α-amylase on 1%/10% (w/v) alginate/starch beads. Adsorbent concentration: 200 g/l; Buffer: 100 mM sodium acetate pH 6.0 with 10 mM calcium chloride; Enzyme concentration 50 U/ml: ○ , enzyme activity profile; ● , protein profile; Enzyme concentration 100 U/ml: □ , enzyme profile; ■ , protein profile.

From literature correlations and estimations for the mass transfer coefficient (k_l) and the binding constant (k_i) it can be derived that diffusion is the rate limiting mechanism in the adsorption reaction [22,23]. The reaction kinetics were modelled using a shrinking core model as described before [10,22]. Description of the adsorption process by this model is appropriate because in the time interval under study (120 min) the adsorption kinetics involve quick saturation of the surface of the immobilized starch particles up to concentrations of 2 U/mg independent of the enzyme liquid concentration [18]. Compared to the diffusion of the enzyme into the bead of crosslinked starch particles immobilized in alginate the saturation of the crosslinked starch particles with the enzyme is a slow process [10,18]. So instead of the saturation value of the isotherm (10 U/mg) a value of 2 U/mg was chosen for q_m in the calculations. The capacity value of the support in terms of U/ml was calculated using the concentration of starch in the beads (Table 6.3).

Simulation revealed that the reaction kinetics are described with diffusion coefficients of 1.0-2.0 × 10^{-11} m^2/s (Table 6.5), both for beads with 0.5% (w/v) and 1% (w/v) alginate. This compares well with previously found

diffusion velocities of 1.0-1.5 × 10^{-11} m^2/s for endo-polygalacturonase in the binding process of this enzyme on alginate beads [10,22] and with the adsorption of β-lactamase on alginate entrapped DEAE-Trisacryl [15], where diffusion was found to be the rate determining step in the adsorption process.

Table 6.5 - Modelling of the adsorption of α-amylase on immobilized starch with a shrinking core model[a].

Adsorbent alginate/starch (% w/v//% w/v)	$c_{b,0}$ (U/ml)	q_m (U/ml)	R (10^{-3} m)	$D_{eff,calc}$ (10^{-11} m^2/s)
0.5/10	50	362	0.61	1.3±0.4
	100	362	0.61	1.4±0.4
0.5/15	50	436	0.61	0.7±0.3
	100	436	0.61	0.8±0.3
0.5/20	50	458	0.88	0.9±0.3
	100	458	0.88	1.0±0.3
1.0/10	50	311	0.93	1.3±0.4
	100	311	0.93	2.6±0.6
1.0/15	50	381	0.87	1.1±0.4
	100	381	0.87	1.8±0.4
1.0/20	50	411	0.96	1.0±0.3
	100	411	0.96	1.6±0.4

[a]: $\epsilon = 0.864$, i.e. hold-up liquid phase $(V_l/[(m_p/\rho_p) + V_l])$.

The change in concentration of alginate causes no significant changes in the calculated diffusion coefficient (Table 6.5). The increase in starch content of the beads (from 192 mg/g to 243 mg/g and 165 mg/g to 218 mg/g respectively, see Table 6.3) also does not result in a significant decrease in the

calculated effective diffusion coefficients. Apparently the possible changes in the effective diffusion coefficient, due to the changes in bead properties, are smaller than 20-30%.

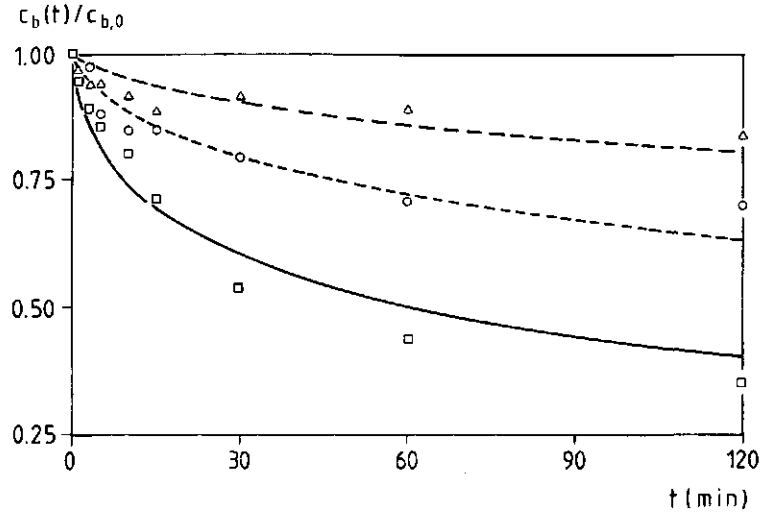

Fig. 6.5 - Modelling the adsorption of α-amylase on alginate/starch copolymers (matrix I). Buffer: 100mM sodium acetate pH 6.0 with 10 mM calcium chloride. Amount of adsorbent: 125 g/l; Experimental results and model calculations for different enzyme concentrations: □ ,——: *50 U/ml;* ○ ,-------: *100 U/ml;* △ ,– – –: *250 U/ml.*

The adsorption of the enzyme by the copolymer beads (matrix I) is given in Fig. 6.5. It shows the adsorption kinetics for different concentrations of enzyme. The relative rate of adsorption decreases at higher enzyme concentrations. A fixed amount of enzyme (approximately 35 U/ml) is adsorbed to the matrix in all cases. The same phenomenon was found for the adsorption of α-amylase on crosslinked starch particles [18]. When the kinetics were modelled with an extended pore diffusion model [22] a sharp decrease of the calculated diffusion coefficient (D_{eff}) (from 3.5×10^{-11} m^2/s to 0.3×10^{-11} m^2/s) was found within the $c_{b,0}$ range of 50-250 U/ml. Both phenomena can be explained by the diffusion resistance due to steric hindrance of the adsorbate covering the adsorbent [22].

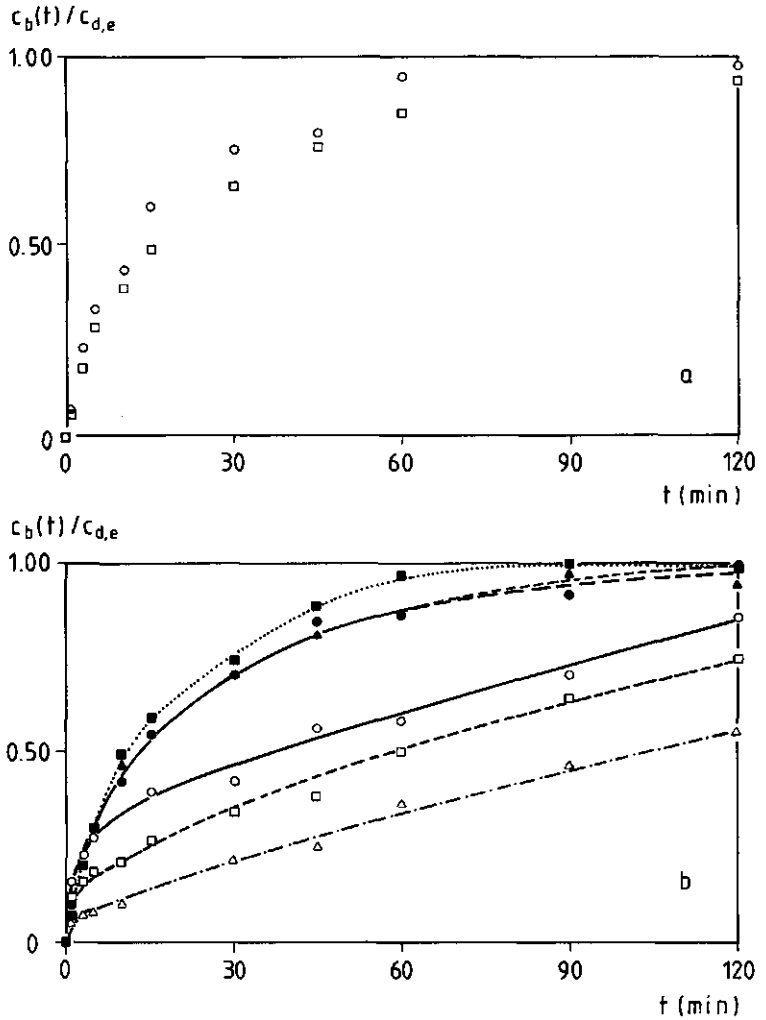

Fig. 6.6 - (a) Desorption of α-amylase from 1%/10% (w/v) alginate/ starch beads with a temperature shift. Buffer: 100 mM sodium acetate pH 6.0 with 10 mM calcium chloride and 20% (v/v) glycerol. Adsorbent: 200 g/l; T = 70 ^{0}C. ○ : $c_{d,e}$ = 40 U/ml; □ : $c_{d,e}$ = 80 U/ml.
(b) Desorption of α-amylase from alginate/starch copolymer beads (matrix 1) with a temperature shift and a limit dextrin buffer. Conditions: 100 mM sodium acetate pH 6.0 with 10 mM calcium chloride. 20 % (v/v) glycerol; T = 70 ^{0}C; 100m mM sodium acetate pH 6.0 with 10 mM calcium chloride and limit dextrins (50 g/l). Adsorbent: 62.5 g/l. ● : $c_{d,e}$ = 45 U/ml, temperature shift; ○ : $c_{d,e}$ = 45 U/ml, limit dextrin; ■ : $c_{d,e}$ = 80 U/ml, temperature shift; □ : $c_{d,e}$ = 80 U/ml, limit dextrin; ▲ : $c_{d,e}$ = 190 U/ml, temperature shift; △ : $c_{d,e}$ = 190 U/ml, limit dextrin.

Desorption

Desorption was accomplished in a 100 mM sodium acetate buffer pH 6.0 with 20% (v/v) glycerol or in a dilute limit dextrin solution (50 g/l). The

dextrin fraction responsible for the elution of the enzyme has a molecular weight of more than 1000 Da. The elution with dextrins has the advantage that process temperatures can be kept at 4 ^{0}C and that thermolabile enzymes can also be recovered. In this way the breakdown of the beads by the enzyme is reduced (Table 6.1) [18].

Table 6.6 - Adsorption and desorption of ∝-amylase on immobilized starch and alginate/starch copolymer beads. Conditions: see Material and Methods.

Enzyme solution	Adsorbent				
	copolymer (I)			immobilized starch	
$c_{b,0}$ (U/ml)	55	102	280	55	106
$c_{b,e}$ (U/ml)	9.5	22	92	15	25
wash (c_w) (U/ml)	0.5	1	2	0.5	1
Desorption ($c_{d,e}$) (U/ml)	42.5[a]	79[a]	160[a]		
	47[b]	78[b]	176[b]	38[b]	75[b]
Recovery	95[a]	100[a]	91[a]		
$(c_{b,e}+c_w+c_{d,e})/c_{b,0}$ * 100	103[b]	99[b]	96[b]	97[b]	95[b]

[a]: *Elution with limit dextrin solution;* [b]: *Elution with temperature shift.*

In Fig. 6.6 desorption of ∝-amylase from beads composed of starch immobilized in alginate and from copolymer beads is given. The data show that the release of enzyme from these beads is slowed down when compared to the (almost instantaneous) desorption from the powder [18]. The release of the enzyme from the beads is slowed down further when limit dextrins are used as an eluent instead of a temperature shift (Fig. 6.6(b)). Whereas the desorption in the latter case appears to be independent of the loading of the particle (Fig. 6.6(a)/6.6(b)), the elution with limit dextrins is clearly dependent on the

Table 6.7 - Adsorption of starch degrading enzymes of different origin on alginate/starch copolymer beads and on beads composed of starch immobilized in alginate[a]. Specific activies of the fractions.

Enzyme	origin		activity		specific activity	
			$c_{b,0}$ (U/ml)	$c_{b,e}$ (U/ml)	starting solution (U/mg)	bound fraction (U/mg)
1.Mycolase[b] (α-amylase)	Aspergillus oryzae	(CP)[e]	13.8	13.6	72	n.d.
		(IS)[f]	13.8	13.6	72	n.d.
2.Maxamyl[b] (α-amylase)	Bacillus licheniformis	(CP)	57	13	160	220
		(IS)	57	19	160	245
3.Dexlo-CL[b,c] (α-amylase)	Bacillus amyloliquefaciens	(CP)	34	22	135	365
		(IS)	34	14	135	330
4.α-amylase[c]	Pig pancreas	(CP)	5.4	1.5	20	17.5
		(IS)	5.4	2.2	20	18
5.α-amylase	Human saliva	(CP)	3.6	0.4	20	80
		(IS)	3.6	1.3	20	45
6.α-amylase/ β-amylase	Barley malt	(CP)	2.0	1.7	33	65
		(IS)	2.0	0.9	33	60
7.β-amylase	Sweet potato	(CP)	104	62	605	780
		(IS)	104	55	605	645
8.β-amylase	Barley malt	(CP)	3.2	2.9	9	8.3
		(IS)	3.2	2.5	9	8.8
9.Amylo-glucosidase	Aspergillus niger	(CP)	3.6	3.6	6	n.d.
		(IS)	3.6	3.6	6	n.d.
10.Pullulanase	Aerobacter aerogenes	(CP)	2.1[d]	2.0[d]	30[d]	n.d.
		(IS)	2.1[d]	2.1[d]	30[d]	n.d.
11.Promozyme[a] (Pullulanase)	Bacillus sp.	(CP)	2.0[d]	2.0[d]	15[d]	n.d.
		(IS)	2.0[d]	2.0[d]	15[d]	n.d.

[a]Reaction conditions: 100 mM sodium acetate pH 6.0; adsorbent: 0.04 g/ml; [b]Mycolase, Dexlo-CL and Maxamyl are products from Gist-brocades; Promozyme is a product from Novo Industries; [c]Desorption is accomplished by elution with glucose syrup (30 mg/ml); [d]Pullulanase activity; [e]CP = copolymer bead; [f]IS = bead composed of starch immobilized in alginate.

concentration of the enzyme on the adsorbent and/or the concentration of limit dextrins in the incubation medium (Fig. 6.6(b)). The desorption velocity is determined by another effect, most likely a change in the desorption reaction parameters (Fig. 6.6(b)), resulting in a slow release of the enzyme in the competitive elution. The equilibrium values ($c_{d,e}$) are the same for the two elution methods (Table 6.6).

In general recovery is over 90% during the process. After 24 h of desorption almost all the adsorbed enzyme is released from the beads (Table 6.6). The specific activities of the desorbed fractions are around 230 U/mg in all cases.

Adsorption of different α-amylases

The adsorption of different commercially available α-amylases and a number of other starch degrading enzymes was investigated at pH 6.0. Table 6.7 summarizes the results obtained in these experiments. The α-amylases of bacterial origin and the β-amylase of sweet potato are readily adsorbed by the matrices whereas the amylase activity of A. oryzae, pullulanase and amyloglucosidase are not adsorbed at the reaction conditions chosen. A temperature shift appears to be a good desorption procedure for heat stable α-amylases like the one from Bacillus licheniformis. The other α-amylases can be eluted by addition of a dilute limit dextrin fraction (50 g/l). In this way more purified preparations are obtained from commercial enzyme preparations. The results are in agreement with and conform to the results obtained earlier with crosslinked starch [13].

6.4 CONCLUSIONS

Isolation of α-amylase on substrate derivatives was described for packed bed applications [12,13]. In this study the development of a bead form affinity adsorbent for α-amylase is described. Two types of adsorbent were developed. Crosslinked starch, synthesized as described previously, is incorporated in an alginate matrix by Ca^{2+}-complexation. In addition the synthesis of a copolymer of alginate and starch is described in which alginate acts as the building frame for the Ca^{2+}-complexed polymer mixture. Subsequently the bead is crosslinked with epichlorohydrin.

Both methods yield affinity adsorbents with comparable affinity characteristics in terms of adsorption equilibrium parameters. The crosslinking conditions have an important influence on the material obtained. The capacities

of the matrices are approximately 12 U/mg. The major factor determining the adsorption reaction velocity is the diffusion velocity of proteins in these hydrogels. Previously diffusion was found to be the limiting factor in the adsorption of endo-polygalacturonase on alginate beads [10]. The adsorption of α-amylase to beads composed of starch immobilized in alginate is characterized by diffusion velocities of 1.0-2.0 × 10^{-11} m^2/s. The adsorption of α-amylase on copolymer beads results in values for the calculated diffusion coefficient of the order of 10^{-12} m^2/s. Although the copolymers have adsorption capacities comparable to immobilized starch, mass transfer is unfavourable in the copolymer matrices. The adsorption process can be improved. Exposure of crosslinked starch to α-amylase results in an improvement of the adsorption capacity of the matrix and improved diffusion of the enzyme in the crosslinked starch powder [18]. Preliminary experiments showed improved binding characteristics for beads composed of starch immobilized in alginate when a crosslinked starch powder which had been exposed to α-amylase was used for immobilization.

Acknowledgement

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NOMENCLATURE

$c_{b,0}$: enzyme concentration at t=0 (U/ml)
$c_b(t)$: enzyme concentration at t=t (U/ml)
$c_{b,e}$: enzyme concentration in liquid phase at equilibrium (U/ml)
c_d : enzyme concentration in liquid phase during desorption (U/ml)
$c_{d,e}$: enzyme concentration during desorption at equilibrium (U/ml)
CR : crosslinking ratio (-)
c_w : enzyme concentration in liquid phase during washing (U/ml)
D_{eff} : effective pore diffusion coefficient (m^2/s)
t : time (s,min)
k_i : rate constant local adsorption (1/s)
k_l : liquid film transport coefficient (m/s)
K_s : Langmuir constant (U/ml)
m_p : mass of matrix (g)
q_m : capacity of support (U/g)
R : radius (m)
T : temperature (^0C)

U : unit of enzyme activity

V : volume (ml)

V_l : volume of the liquid phase (ml)

Greek symbols

ϵ : hold-up liquid phase (-)

ρ_p : density of wet support (g/ml)

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CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTION

In this thesis the development of affinity matrices for endo-polygalacturonase and α-amylase has been decribed. Aspects of the application in isolation and purification processes will be discussed in this chapter.

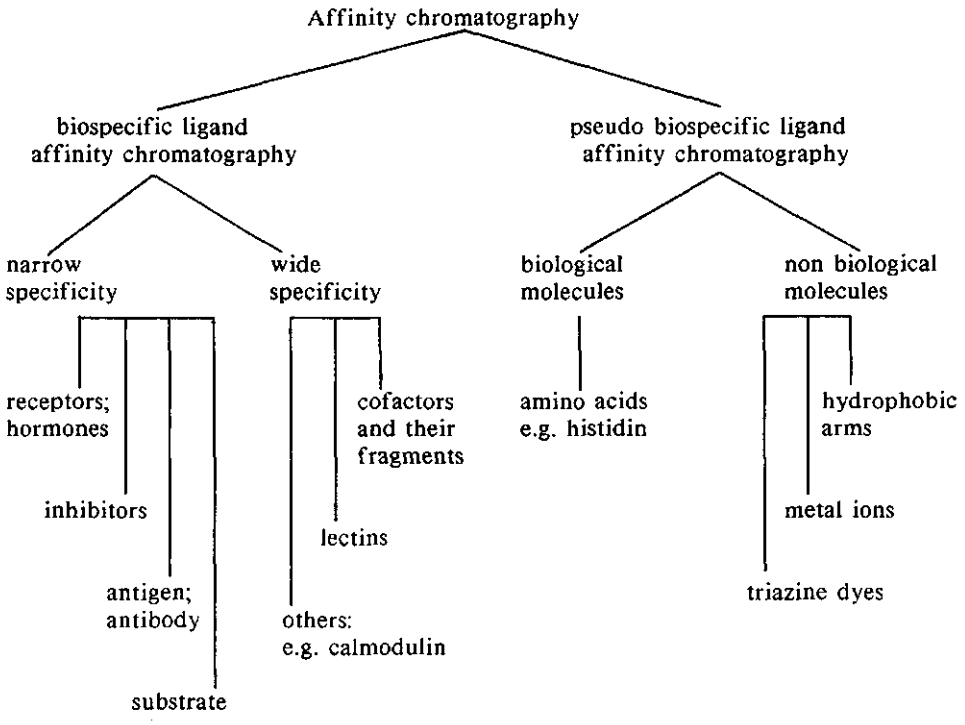
7.2 AFFINITY SEPARATION PRINCIPLES

Adsorption characteristics

Affinity separation techniques are characterized by the use of the recognition between an immobilized ligand and the molecule to be isolated. The field of affinity chromatography is very broad resulting in the exploitation of numerous, to a more or less extent specific, interactions for the extraction of biological molecules [1]. Scheme 7.1 shows an overview of affinity separation systems based on the affinity ligand used. Biospecific and pseudo-biospecific affinity systems are distinguished, the latter category containing the interactions of biomolecules with hydrophobic ligands, dyes, metals or sulphur as reviewed elsewhere [2,3,4].

Highest selectivity is usually obtained in the systems on the left-hand side of Scheme 7.1. Here receptor-hormone, antigen-antibody, enzyme-inhibitor and enzyme-substrate interactions are situated. The underlying phenomenon for this kind of interactions is the specific interaction between the ligand and the ligate via a specific binding site, based on a biological interaction. They are characterized by a strength of binding with K_S values between 10^{-5} and 10^{-12} mol/l. The affinity separation techniques, described in this thesis, are based on enzyme-substrate interactions (Chapters 3 and 5).

The interaction of endo-polygalacturonase with alginate is characterized by approximately the same K_S value as the interaction of α-amylase with crosslinked starch (10^{-6} mol/l). The specific activity of the purified fraction is 120 U/mg when endo-polygalacturonase was purified from a commercial enzyme sample with the beaded alginate. Further purification on an ion-exchanger yielded an activity of 580 U/mg. The selectivity of the adsorbent for α-amylase appears to be very good. Irrespective of the source from which the enzyme is recovered (commercial enzyme samples, clarified fermentation media) an enzyme fraction with a specific activity of 220 U/mg is obtained. When assayed on Poly Acryl Amide Gel Electrophoresis it appears that the enzyme is about pure (> 95%). This enables a one step purification of complex mixtures.



Scheme 7.1 - Affinity chromatography - classification on basis of type of ligand (adapted from ref. 1).

Synthesis of affinity adsorbents

Bio-affinity adsorbents are usually designed by coupling a ligand (a biomolecule) to an inert matrix resulting in water insoluble complexes that can be used as carrier in the extraction process. The functional groups for ligand immobilization are listed in Table 7.1.

As coupling gels mostly (activated) dextrans, agaroses, polyacrylamide and silicas are used [5]. These matrices are commercially available. For large scale purification of relatively cheap products such as endo-polygalacturonase and α-amylase these kinds of activated matrices can not be applied in most cases because of cost reasons. Additional to the cost of the matrix itself, the price of the common affinity matrix is determined also by the activation process which the non-reactive matrix requires prior to coupling the ligand. This means that a production method for a suitable matrix will always consist of a two step reaction scheme. In the first step a functional group is coupled to an inert matrix, followed by coupling of a ligand to the activated carrier.

Table 7.1 - Biomolecules as ligands in affinity chromatography and functional groups on the adsorbent via which they are immobilized.

ligand	functional group
protein, peptide, amino acid	amino-, carboxyl- and thiol-groups
sugar(s)	hydroxyl-, amino-, and carboxyl-groups
(poly)nucleotides	amino-group
coenzymes, cofactors, antibiotic, steroids etc.	amino-, carboxyl-, thiol or hydroxyl-groups

The method developed in this thesis does circumvent these problems. The affinity matrices are prepared by a direct complexation and/or crosslinking of substrates or substrate analogues which yields the ready for use affinity matrix in one step. The method for adsorbent preparation is extremely versatile which enables the possibility to synthesize affinity adsorbents for a number of polymer hydrolyzing enzyme activities. Furthermore the building blocks are low-cost products, viz. alginate and starch.

Affinity matrices and affinity chromatography of polysaccharide degrading enzymes

Application of affinity chromatography in large-scale downstream processing is rare up to now. The combination of high specifity and high stability of the adsorbent in purification processes is hard to realize. For most systems isolation of the necessary ligand (see Scheme 7.1) is a laborious procedure and an expensive operation resulting in high adsorbent and subsequent process costs [6].

The aim of this study was the development of cheap affinity systems that can be applied on a large scale. The development of the adsorbents described

in this thesis combine a number of advantages in this respect. The enzymes under study have low cost polymers as their substrate. This property allows the synthesis of an affinity adsorbent by a crosslinking reaction of the substrate with an appropriate reagent. The product of this reaction is a water insoluble material with affinity for the enzyme.

7.3 APPLICATION OF AFFINITY MATRICES

The most common way to apply affinity adsorbents in a purification process is the use of packed bed chromatography. For commercial applications it can suffer from limitations inherent to the method (e.g. low flow rates, compressibility of gel beads, mass transfer limitations) as well as to the adsorbent (expensive). As a result affinity chromatography needs clarified and relatively pure process streams making it usually the final step of a purification process. Direct isolation of a product from a fermentation broth allows the elimination of a number of unit operations from the purification process and may yield a cheaper overall procedure [7]. The beads developed in the work described in this thesis allow applications in fermentation broths.

The second aspect in this research was the development of processes for the direct application of affinity systems on fermentation broths in continuous extraction processes. Several separation principles have recently been introduced. The most important developments are fluid bed columns [8,9], membrane cross-flow filtration [10], reversed micellar extraction [11] and two-phase aqueous extraction [12]. The development of affinity based interactions in these fields is also evolving [13,14].

Fluid bed separations

The adsorbents described in Chapters 2 and 6 can be readily used in fluid bed applications [15]. Counter-current fluid bed separations are described in literature [16]. A process scheme is given in Fig. 7.1. The use of fluid bed affinity adsorption combines several advantages:

- the possibility to process a fermentation broth including the solids;
- the low pressure drop;
- the short residence time of the biomolecule in the adsorbed state;
- the selectivity of the affinity adsorbent;
- the continuous process.

The major advantage of this process is the extraction selectivity. The

enzyme can be recovered in one process step, yielding a pure enzyme preparation. This is a unique property which allows a direct isolation of the protein thus replacing a number of unit operations now used to extract the enzyme from the process medium.

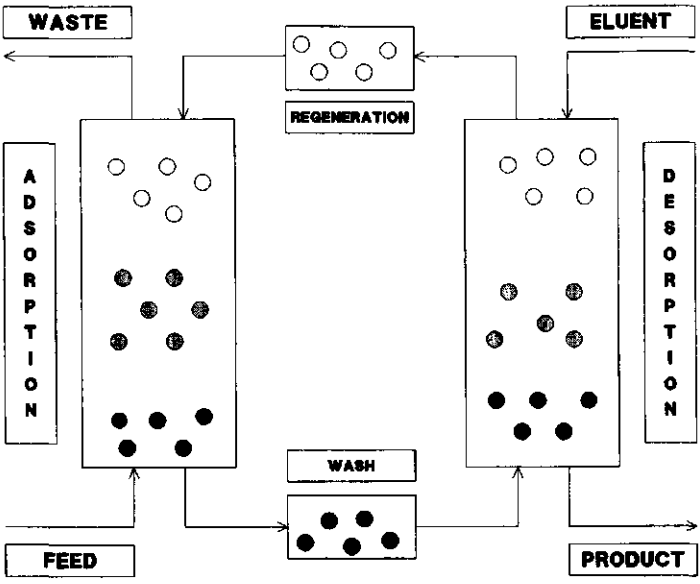


Fig. 7.1 - Scheme for a continuous adsorption/desorption process.

There are also disadvantages in using the bead type of matrices. Equilibrium times in the adsorbents (approximated diameter 1.5×10^{-3} m) can be estimated to be larger than 15 h due to the diffusion determined mass transfer. This can be overcome by partial loading of (the outside of) the bead, although this will result in an inefficient use of the adsorbent in the process. A decrease of the particle size will reduce this problem as diffusion times are dependent on the square root of the particle diameter. Developments in fluid bed technology and matrix preparations [17-20] can be combined to prepare small magnetic affinity adsorbents on the basis of the principles described in this thesis to be used in a magnetically stabilized fluid bed [20].

The developed adsorbents contain a relatively large amount of water. When leaving the adsorption reactor, the "liquid phase" of the adsorbent does contain almost no enzyme. The concentration increase which can be obtained in the counter current desorption extractor depends on the number of stages

in the extractor, the required extraction efficiency and the flow ratio of the phases. The process is characterized by an extraction factor (L= (k×U)/D) in which the distribution coefficient (k) equals one. In Chapter 6 it is shown that the overall concentration of the enzyme in the developed adsorbents in the adsorbed state is 2500 U/ml. This means that the concentration of the back-extraction (desorption) fluid coming out of the desorption column (see Fig. 7.1) will have a maximum concentration of about 2500 U/ml α-amylase when a fully loaded adsorbent consisting of immobilized starch is used. Although this represents a protein concentration of approximately 10 g/l it also shows the upper limit for the concentration to be reached using this kind of matrices.

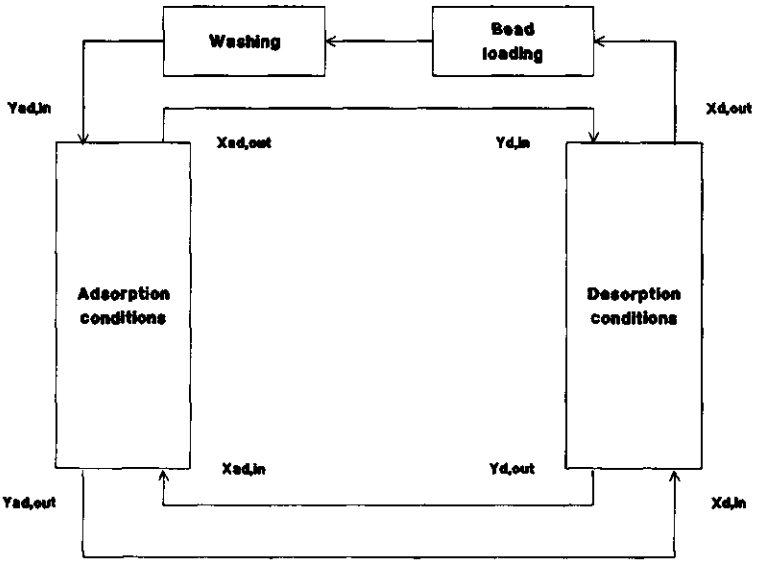


Fig. 7.2 - Process scheme for concentration of α-amylase in two counter-current extractors.

By applying a process scheme as shown in Fig. 7.2, theoretically the concentration of the enzyme in the product stream can be increased for the α-amylase purification. This system has the advantage that no change in the reaction medium is needed under adsorption and desorption conditions due to the fact that desorption can be accomplished by a temperature shift (see Chapter 4). This principle, combined with the high thermal stability of the enzyme, can be used to obtain the desired increase of concentration in the

product stream. By exchanging the liquid phase of the adsorbent under adsorption conditions against the enzyme solution obtained in the desorption step (see Fig. 7.2) a bead with an increased enzyme concentration is obtained. Subsequently the enzyme can be recovered by back-extraction in a second extractor under desorption conditions (temperature raise) (see Fig. 7.2) resulting in an increase of the enzyme concentration in the product stream. The desorption fluid is cooled to 4 ^{0}C and can be used again to be exchanged against the adsorbent liquid phase in the extractor under adsorption conditions.

Limitations in this process are the low distribution coefficient, the fact that the adsorbent/liquid ratio is limited to a certain range due to practical restrictions (hold-up of particles in the fluidized bed) and the fact that the liquid flow in the extractor under adsorption conditions is coupled to the liquid flow in the extractor under desorption conditions. Model calculations reveal that under realistic process conditions a concentration factor of 2-3 can be realized starting with fully saturated beads.

Affinity membrane filtration

Another way to obtain a high enzyme concentration in the product stream is by using smaller adsorbent particles with higher enzyme concentrations (see Chapter 5). When saturated matrices of this type are extracted in a counter-current extraction process much higher concentrations of enzyme can be achieved. This can for example be achieved in a membrane filtration set-up.

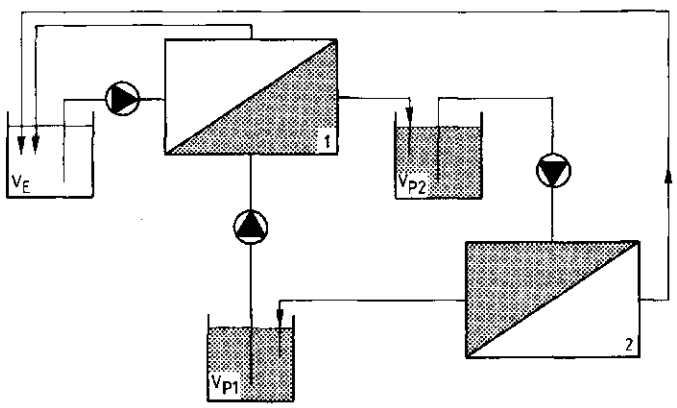


Fig. 7.3 - Experimental set-up for affinity membrane filtration. V_E: Enzyme reservoir; V_{P1}, V_{P2}: Adsorbent suspension reservoirs. Volumes: External (enzyme) circuit: 200 ml; Internal (adsorbent) circuit: 400 ml; Adsorbent concentration: 20 mg/ml. Temperature: 4 ^{0}C.

The underlying idea of affinity membrane filtration is the use of small affinity adsorbents combined with a membrane filtration step. Its basic principle is that the desired protein will pass through the membrane; the adsorbent and the adsorbent/protein complex wil not. By transporting the adsorbent through a number of membrane modules conditioned for adsorption and desorption respectively, the protein can be isolated from a fermentation broth. Advantages of this process set-up are:

- the possibility of cell separation by a (microporous) membrane;
- continuous process (transport of adsorbent);
- separation of low molecular weight impurities by filtration;
- selective recovery.

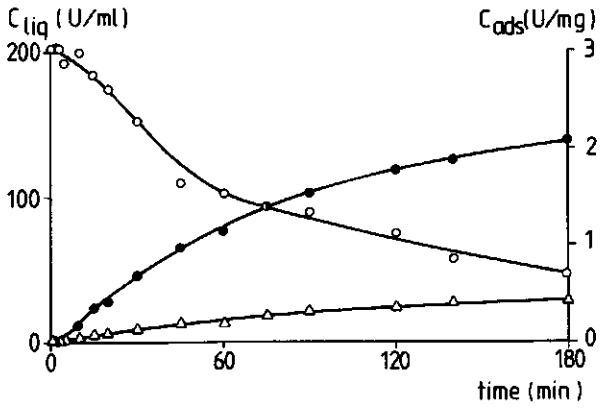


Fig. 7.4 - Adsorption of ∝-amylase by affinity membrane filtration.
○ : Enzyme concentration in V_E (U/ml); △ : Enzyme concentration liquid phase internal circuit (U/ml); ● : Enzyme loading of adsorbent (U/mg).

The application of crosslinked starch in affinity membrane filtration was realized. Alpha-amylase of B. licheniformis was purified from a commercial enzyme sample. A batch adsorption reaction was performed in two membrane modules through which the adsorbent suspension (20 mg/ml) was recirculated

(Fig. 7.3). In the first membrane module (0.2 μm PVDF membrane, ENKA, FRG) the enzyme permeates to the adsorbent by applying a transmembrane pressure (flux: 11.5 l/h.m^2.bar). The internal liquid volume of the reactor was kept constant by introducing a second module (0.2 μm ceramic membrane, sfec, France) by which the liquid phase could permeate back to the enzyme reservoir. The enzyme was purified by a factor 1.8 (specific activity: 230 U/mg) with regard to the protein content relative to the starting solution. The recovery on basis of enzyme activity was 91%. The concentration profiles in the reaction compartments are given in Fig. 7.4. The system was operated for six hours without any problems related to clogging of the membrane modules or fouling of the membrane surface.

An advantage of this system is the possibility to use crosslinked starch without further modification. Furthermore the improved mass transfer characteristics of the crosslinked starch compared to the beaded adsorbents are exploited.

Development of water soluble polymers carrying an affinity ligand [21] or the attachment of the ligand to the membrane can improve binding kinetics by reduction of mass transfer limitations [22].

7.4 CONCLUSION

It can be stated that affinity chromatography is a promising technique to purify enzymes on a large scale. Selective adsorbents have been developed which are easy to prepare. They can be used extensively in a purification process, yielding enzyme fractions with high specific activity. The adsorbent preparation may be also applicable to other enzyme systems.

Application of the adsorbents in fermentation media is possible. Fluid bed technology for alginate particles and alginate included adsorbents is described [15,23]. The major obstructions are the hindered mass transport due to diffusion resistance and the low concentration of the enzyme in the product stream. The first problem can be overcome by reduction in particle size and application of the adsorbent in affinity membrane filtration. The second problem can be overcome for the α-amylase system by rational process design.

The enzyme products which are now on the market can be purified further with the developed techniques. Whether this will be useful and important will be decided in the future. Probably law demands and regulations and market needs will make development of more purified enzyme preparations necessary. Tailor made enzyme preparations, free of side activities, may be

very useful in enzyme applications in for example the food industry. As a result this will make large scale affinity purification processes profitable compared to the conventional large scale purification processes. Further research and a cost analysis will decide if it is economically feasible to introduce this kind of purification technology in industrial processes.

NOMENCLATURE

L : strip factor (-)

k : distribution coefficient (-)

U : flow rate uptaking phase (m^3/s)

D : flow rate delivering phase (m^3/s)

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SUMMARY

The objective of this study was the development of affinity matrices to isolate and purify a number of polysaccharide degrading enzymes and the application of these adsorbents in the large-scale purification of the enzymes from fermentation broths. Affinity adsorbents were developed for endo-polygalacturonase and α-amylase.

The isolation of two of these enzymes was realized using the specific affinity of the enzymes for the corresponding substrates, viz. pectate and starch. Normally interaction between an enzyme and its substrate is accompanied by hydrolysis of the polymeric substrate, resulting in total biodegradation. By specific modification of the substrate it is possible to obtain adsorbents which are capable of binding the enzyme while being resistant against biodegradation.

Pectate is the natural substrate for endo-polygalacturonase. Alginate, a substrate analogue for pectate, is able to bind endo-polygalacturonase while it is not hydrolyzed by the enzyme. Rigid beads can be obtained by calcium complexation of the alginate. The pH and ionic strength of the incubation medium influence the strength of the interaction between endo-polygalacturonase and alginate beads. Adsorption end desorption can be controlled by these two parameters. In this way the enzyme can be isolated and purified from complex mixtures. The adsorbent can be regenerated at least a hundred times in a continuous process (Chapter 2).

The adsorption of the enzyme to the matrix was subject of further study. By determining relevant mass transport parameters such as adsorption equilibrium parameters, diffusion coefficients and rate parameters it appeared to be possible to describe the adsorption process in mathematical terms. The velocity of adsorption is determined by the diffusion velocity of the enzyme in the beads and not by the reaction kinetics of the complex formation. The velocity of the desorption process is also determined by the diffusion velocity of the enzyme out of the bead (Chapter 3).

The most important substrate for α-amylase is starch. Alpha-amylase is used on a large scale for the enzymic conversion of starch into limit dextrins and other oligosaccharides. By means of a chemical crosslinking procedure of starch an adsorbent is obtained which is capable of binding the enzyme while it is degraded only to a limited extent. The adsorption and desorption characteristics of the interaction between enzyme and adsorbent were studied. It appears that the enzyme has the highest affinity for the adsorbent at the pH where it has its maximum catalytic activity. The interaction is biospecific

and this principle allows a very selective isolation of the enzyme. The interaction between enzyme and adsorbent is essentially insensitive to changes in ionic strength of the medium. Desorption can be accomplished by a shift of pH or a raise in temperature of the incubation medium (Chapter 4).

The adsorption characteristics were further evaluated. Continuous use of the adsorbent in an isolation process of α-amylase results in a slow biodegradation of the matrix. This effect is accompanied by an increase of capacity of the adsorbent for the enzyme. It appears that the adsorbent can be used repeatedly for the isolation of the enzyme; the biodegradation is made up for by an improved mass transfer of the enzyme into the matrix combined with the increased capacity. The rate of adsorption is determined by the diffusion rate of the enzyme into the porous gel (Chapter 5).

For the direct application of adsorbents in fermentation broths a number of techniques have been proposed. One of these is the use of a fluid bed column. This imposes a few demands on the density and the diameter of the adsorbent. Particles, suitable for use in fluid bed columns were developed by inclusion of crosslinked starch in alginate particles and by the preparation of an alginate/starch copolymer bead. The adsorption characteristics of these adsorbents are comparable with those of crosslinked starch (Chapter 6).

In conclusion it can be stated that affinity separations for endo-polygalacturonase and α-amylase prove to be a selective process with good potential for a one step purification of these enzymes from a fermentation broth. In addition the procedure of adsorbent preparation offers good opportunities to prepare affinity adsorbents for other hydrolases.

This study was performed in a partnership between the Department of Food Science (Food and Bioprocess Engineering Group and Food Chemistry and Food Microbiology Group) and the Department of Genetics. The project was financed by the Netherlands Technology Foundation (STW).

SAMENVATTING

Het doel van het in dit proefschrift beschreven onderzoek was de ontwikkeling van affiniteitsadsorbentia voor een aantal polysaccharide splitsende enzymen en de toepassing ervan bij de opwerking van deze enzymen uit fermentatiemedia. Daartoe zijn adsorbentia ontwikkeld voor twee enzymen, endo-polygalacturonase en α-amylase.

De isolatie van de genoemde enzymen is bewerkstelligd door gebruik te maken van de specifieke affiniteit van de enzymen voor hun substraat, te weten pectaat en zetmeel. Normaal gaat de interactie van de enzymen met deze substraten gepaard met hydrolyse, uiteindelijk resulterend in een totale afbraak van het substraat. Door speciale modificaties in de substraten aan te brengen kunnen matrices verkregen worden die de enzymen kunnen binden en goed bestand zijn tegen enzymatische afbraak.

Pectaat is het substraat voor het enzym endo-polygalacturonase. Alginaat, een qua structuur sterk op pectaat gelijkend molecule is in staat endo-polygalacturonase te binden, maar wordt niet gehydrolyseerd. Om vaste deeltjes te krijgen is het alginaat met calcium gecomplexeerd tot een bolvorm. De pH en de ionsterkte van het adsorptiemedium blijken een grote invloed te hebben op de binding van endo-polygalacturonase aan alginaatbolletjes. Adsorptie en desorptie van het enzym aan de matrix blijken geregeld te kunnen worden door een juiste instelling van de genoemde parameters. Op die manier blijkt het mogelijk het enzym te extraheren. Het adsorbens blijkt zeer lang bruikbaar te zijn in een continu adsorptie-/desorptieproces (Hoofdstuk 2).

De adsorptie van het enzym aan de matrix is verder bestudeerd. Door het bepalen van relevante stofoverdrachtsparameters zoals adsorptie evenwichts-parameters en de diffusiecoefficiënt kan het adsorptieproces modelmatig worden beschreven. De snelheid van adsorptie van endo-polygalacturonase aan de alginaatdeeltjes wordt bepaald door de diffusiesnelheid van het enzym in de bolletjes en niet door de bindingssnelheid van het enzym aan het alginaat. Ook het desorptieproces wordt bepaald door de diffusiesnelheid van het enzym in de matrix (Hoofdstuk 3).

Het belangrijkste substraat voor het enzym α-amylase is zetmeel. Alpha-amylase wordt grootschalig gebruikt bij de industriële vervloeiing van zetmeel tot glucosestropen. Door een chemische verknoping van de zetmeelketens kan een adsorbens verkregen worden dat in staat is het enzym te binden, maar dat slechts in geringe mate wordt afgebroken.

De adsorptie- en desorptiekarakteristieken van de enzym/adsorbens-interactie zijn bestudeerd. Daarbij blijkt dat het enzym bij een pH waarbij het

ook zijn maximale katalytische aktiviteit heeft de sterkste interactie vertoont met het verknoopte zetmeel. Op basis van verdere experimenten kon worden aangetoond dat de interactie biospecifiek is en dat het mogelijk is het enzym zeer selectief aan de matrix te binden. De binding van het enzym aan de matrix is vrijwel ongevoelig voor de ionsterkte van het medium. Het blijkt dat het enzym van het adsorbens kan worden gedesorbeerd door een verschuiving van de pH naar een gebied waar de katalytische aktiviteit van het enzym gering is. Een andere methode is een verhoging van de temperatuur van het reactiemedium (Hoofdstuk 4).

De adsorptiekarakteristieken van dit systeem zijn verder bestudeerd. Het blijkt dat bij continu gebruik van het adsorbens voor de opwerking van α-amylase de matrix langzaam door het enzym afgebroken wordt. Dit gaat echter gepaard met een toename van de capaciteit van het adsorbens voor het enzym. Op deze manier is het adsorbens toch gedurende een geruime tijd bruikbaar in een continu opwerkingsproces. Daarbij wordt de afbraak ruim gecompenseerd door een verbeterd massatransport van het enzym in de drager, gecombineerd met een toename van de capaciteit van de matrix voor het enzym. De adsorptiesnelheid van α-amylase aan het verknoopte zetmeel wordt bepaald door de diffusiesnelheid van het enzym in de korrel (Hoofdstuk 5).

Om een directe toepassing van affiniteitstechnieken in fermentatiemedia te verwezenlijken staan een aantal technieken ter beschikking. Eén daarvan is het gebruik van een fluïde bed. Het gebruik hiervan stelt eisen aan de dichtheid of de grootte van het te gebruiken adsorbens. Bruikbare deeltjes voor fluïde bed toepassingen zijn ontwikkeld door insluiting van chemisch verknoopt zetmeel in alginaatdeeltjes en door de bereiding van een copolymeer van alginaat en zetmeel. De adsorptiekarakteristieken van deze matrices komen overeen met die van verknoopt zetmeel (Hoofdstuk 6).

Concluderend kan gesteld worden dat affiniteitsextractie van endo-polygalacturonase en α-amylase een selectief proces is dat een enorm potentieel biedt deze eiwitten te zuiveren en te concentreren in één stap. Verder bieden de ontwikkelde technieken voor adsorbensbereiding de mogelijkheid analoge adsorbentia te bereiden voor andere hydrolases.

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CURRICULUM VITAE

Wim Somers werd op 20 oktober 1958 geboren te Roosendaal en Nispen. In 1977 behaalde hij het diploma Atheneum B aan het St. Pauluslyceum te Tilburg. In hetzelfde jaar begon hij aan de studie Scheikunde aan de Katholieke Universiteit Nijmegen. In 1985 studeerde hij af met als hoofdvak Biochemie (Prof. Dr. J.J.H.M.M. de Pont en Prof. Dr. F. Daemen), Chemische Cytologie (Prof. Dr. Ch.M.A. Kuyper), Chemische Microbiologie (Prof. Dr. Ir. G.D. Vogels), Kunstgeschiedenis van de Middeleeuwen (Dr. H.A. Tummers) en Proceskunde (Prof. Dr. Ir. K. van 't Riet). Van 1985 tot 1990 was hij werkzaam bij de Vakgroep Levensmiddelentechnologie (Secties Proceskunde en Levensmiddelenchemie en levensmiddelenmicrobiologie) en de Vakgroep Erfelijkheidsleer van de Landbouwuniversiteit Wageningen in een STW project. Vanaf 1991 is hij werkzaam bij de afdeling Biotechnologie van de Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO-Voeding).