Affinity Purification

of Polysaccharide Degrading Enzymes

with Crosslinked Substrates

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Stellingen

1. Aan de hand van de oxydatie van cyclohexeen met een secundair alkylperoxide is nogmaals duidelijk geworden dat het slechts toepassen van gaschromatografische analyse tot een verkeerde interpretatie van de produktsamenstelling en het reactiemechanisme kan leiden. Wanneer thermisch instabiele reactieprodukten verwacht kunnen worden moet men zich bedienen van niet-destructieve analysemethodieken als HPLC en NMR.

   Sarneski, J.E., Michos, D., Holden Thorp, H., Didiuk, M., Poon, T., Blewitt, J., Brudvig G.W., en Crabtree, R.H. (1991) Tetrahedron Lett. *32*, 1153-56.

2. De bewering van Masaki *et al.* dat de reactie van een nucleofiel agens met 3,4,5,6-tetrahydro-2H-azepine-7-ol waterstofsulfaat via een aanval op het centrale koolstofatoom slechts kan leiden tot een op de 7-plaats gesubstitueerd 3,4,5,6-tetrahydro-2H-azepine derivaat houdt geen rekening met de mogelijkheid dat reactie van het laatstgenoemde derivaat met zwavelzuur alsnog ϵ-caprolactam op zou kunnen leveren.

   Masaki, M., Uchida, M., en Fukui, K. (1973) Bull. Chem. Soc. Jap. *46*, 3174-79.

3. Wanneer de conversie van een uitgangsprodukt met het begrip "opbrengst" wordt verward, worden resultaten onvolledig en te rooskleurig weergegeven.

   McKinney, R.J., Hydrocyanation of conjugated 2-alkenoates, PCT Patent, DuPont de Nemours and Comp., WO 91/17140, 14 november 1991.

4. De door Sasaki *et al.* beschreven hydroxylering van benzeen toont aan dat de hoge produkt-selectiviteit, die in het algemeen vaker bij lagere reactie-conversies wordt gevonden, mede te danken kan zijn aan een lagere kwaliteit van de relevante analyses.

   Sasaki, K., Ito, S., en Kunai, A. (1990) in New Developments in Selective Oxidation (Centi, G., en Trifiro, F., Ed.), pp. 125-131, Elsevier, Amsterdam.

5. De verklaring van Viersen *et al.* voor het verschuiven van λ_{max} bij gelijkblijvende adsorptie-intensiteit impliceert identieke molaire extinctie-coëfficiënten voor de sterk verschillende Cu-tmed complexen die in het evenwicht zijn betrokken, hetgeen hoogst onwaarschijnlijk is. Een betere verklaring is het optreden van waterstofbruggen tussen tmed en bruggende hydroxides in polynucleaire kopercomplexen.
Viersen, F.J., Challa, G., en Reedijk, J. (1989) Rec. Trav. Chim. Pays-Bas *108*, 167-171.

6. De weergave van log K ten opzichte van de ringgrootte (C_{14}, C_{15} etc.) waarbij de meetpunten onderling worden verbonden suggereert ten onrechte dat er gebroken koolstofatomen in een macrocylische ring voor kunnen komen.
Hancock, R.D., en Martell, A.E. (1989) Chem. Rev. *89*, 1875-1914.

7. De recente inspanningen op het gebied van recycling van polyamides in textiele garens ten spijt, lijkt de meest efficiënte manier van recycling van polyamides toch door instanties als het Leger des Heils bewerkstelligd te kunnen worden.

8. De trend om in Nederlandse bedrijven kennisdragers vervroegd te pensioneren, zal er toe leiden dat in de toekomst het zwarte garen weer vaak zal worden uitgevonden.

9. De nieuwe, uiterst belabberde, zaalvoetbalspelregels die in 1992 zijn ingevoerd, zullen door een sterk vergrote kans op blessures de gezondheidskosten in Nederland nog verder doen stijgen.

Stellingen behorende bij het proefschrift "Affinity purification of polysaccharide splitting enzymes with crosslinked substrates" door H.J. Rozie. Wageningen 11 december 1992.

Contents

Chapter 1

Introduction

The last decades have shown an increased application of enzymes in industrial processes. An important part thereof concerns polysaccharide splitting enzymes that are used in food industry. Examples can be found in the degradation of starch (α-amylase and glucoamylase), the clarification of fruit juices (pectinase) and the maceration and liquefaction of agricultural products. The source of these bulk enzymes is practically always a microbial one. Often, the applied enzyme preparations are rather complex mixtures of enzymes and the composition of these mixtures is not thoroughly optimized for their applications. In fact, the optimum composition of enzyme preparations as cocktails to carry out certain enzyme processes is nowadays often unknown. To improve this, further purification and characterization of the individual enzymes is necessary. This has to result in enzyme formulations of a constant quality that are tailor-made towards their applications. Such a mixture of purified enzymes of a well-known composition complies also better with the modern quality standards of chemical and biochemical products. The costs of the existing purification techniques that offer high selectivity are, however, an economical bottle-neck. New methods with regard to isolation and purification of industrial enzymes are needed. The aim of this work was to find economically favourable purification methods for several polysaccharide splitting bulk enzymes based on affinity interactions.

Affinity interactions

An important area of interest covers purification techniques based on affinity interactions between enzymes and specific ligands. This approach potentially offers a quick one-step procedure to isolate specific enzymes from complex mixtures. An additional advantage is that with this technique one can concentrate very dilute enzyme solutions. However, suitable off-the-shelf affinity adsorbents are usually not available, in which case an adsorbent has to be synthesized. Natural or synthetic ligands (e.g. known enzyme inhibitors, substrates, dyes) are in most cases bound through a spacer arm to an inert solid support such as Sepharose or Eupergit C (Janson, 1984; Lowe, 1984). A huge number of these systems have been developed in the last decades, including also antibody-based adsorbents (Chase, 1984). Even if

natural proteinaceous ligands are available, a main disadvantage is their general instability and high cost. Therefore synthetic ligands were developed and especially dye ligands were used widely in laboratory purifications (Clonis *et al.*, 1987). The problems of dye leakage and poor reproducibility due to byproducts coproduced in the dye-manufacturing process, were disadvantages that did make commercialization on a larger scale difficult. Although improvements were made in this respect (Jones, 1991) it is generally felt that carrier-ligand adsorbents prepared with natural or synthetic ligands were less suitable for large-scale applications with relatively cheap bulk-enzymes. The adsorbent material is often rather costly which can be caused by expensive supports, ligands and chemicals for attachment.

Another approach to obtain an affinity adsorbent is to modify the inexpensive substrate of a polysaccharide splitting enzyme. In that way support and ligand are indistinguishable. The synthesized adsorbent has to comply to the following conditions:

a) The modified substrate should be insoluble since it must be separated from the enzyme mixture.
b) The modified substrate should retain most of its original affinity towards the enzyme.
c) The modified substrate should be stable against enzymatic degradation.
d) The adsorbed enzyme must be easily desorbed from the modified substrate.

The conditions listed are only the main ones. For an eventual industrial application an affinity adsorbent also needs good diffusion properties, mechanical stability and a long life-time.

Crosslinking with epichlorohydrin

The four conditions described above are guidelines in the first stage of developing a new adsorbent for a specific hydrolase by modifying the natural substrate. Insolubility or immobilization of the modified substrate and simultaneous protection against enzymatic degradation are achieved by crosslinking the polysaccharide with a bifunctional agent, such as epichlorohydrin. With starch for instance, this agent reacts with the hydroxy-groups of the glucose monomers as if it were a diepoxide (Fig. 1). Two monomers are thus connected through a glyceryl bridge and a three-dimensional network will be formed which appears as a solid or gelly structure. The required

aqueous alkaline solution will lead also to side reactions as the formation of a monoglyceryl ether derivative which will not contribute to the formation of the network (Flodin, 1962). Thus, except for a change in the three-dimensional structure the derivative consists of sugar moieties that are partly substituted with glyceryl monoethers or etherbridges.

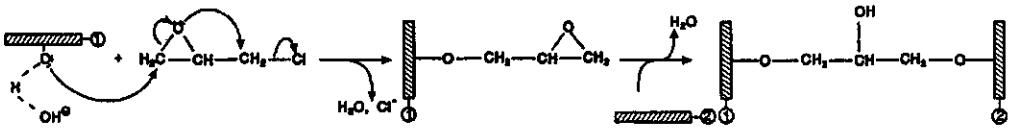

Fig. 1 Mechanism of the crosslinking reaction of polysaccharides with epichlorohydrin that results in a glyceryl diether bridge.

Affinity purification with crosslinked polysaccharides

Noticeable is that also in nature the substrates of the polysaccharide splitting enzymes are in some cases stored in such a way that affinity to the enzymes remains while at the same time protection is provided against enzymatic degradation. Examples thereof are found in the adsorption of α-amylase on crystalline starch (Leloup *et al.*, 1991) and the adsorption of cellulases on wood (Fujishima *et al.*, 1989). When crosslinked polysaccharides are used as an adsorbent the enzyme has to recognize parts of the modified polymer chain as its natural substrate but should not degrade this chain into oligomers which would partly or completely solubilize that particular adsorbent. It appeared that with crosslinking of polysaccharides it is not easy to fulfil both requirements completely. When for example starch was crosslinked slightly with epichlorohydrin, the materials were easily degraded and such products can even be used for determination assays of α-amylase (Mateescu & Schell, 1983 and Ostafe *et al.*, 1984). When polysaccharides were crosslinked more thoroughly and used as enzyme adsorbents they were in general also susceptible to degradation. However, on laboratory scale different crosslinked adsorbents were synthesized and used to purify a certain amount of enzyme. An example is the purification of a fungal cellulase on crosslinked cellulose (Weber *et al.*, 1980). The purification of other endo-enzymes (polygalacturonase, α-amylase and xylanase) are discussed in the next chapters of this thesis. In addition to endo-enzymes also some fungal polysaccharide degrading exo-

enzymes, such as α-L-arabinofuranosidase and 1,2-α-mannosidase, have been purified on crosslinked polysaccharides, namely on crosslinked arabinan (Waibel *et al.*, 1980) and on crosslinked mannan (Tanimoto *et al.*, 1986), respectively.

In this thesis the development of adsorbents for three different types of industrially important polysaccharide splitting enzymes is described. The purification of polygalacturonases with crosslinked substrate was studied before by Rexová-Benková and Tibensky (1972). Also in these studies adsorbent degradation was inevitable. In Chapter 2 another method for purifying polygalacturonases is discussed using a modified substrate analog as an undegradable adsorbent. The crosslinking of soluble starch and the adsorption of bacterial α-amylases on such an adsorbent is studied thoroughly and described in chapters 3 and 4. A much more complex adsorbent results from crosslinking of a xylan heteropolymer. The development of a suitable xylanase adsorbent is discussed in chapters 5 and 6.

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Chapter 2

Rozie, H., Somers, W., Bonte, A., Visser, J., van 't Riet, K., and Rombouts, F.M. (1988). Adsorption characteristics of endo-polygalacturonase on alginate beads. Biotechnol. Appl. Biochem. 10(4), 346-58.

Adsorption Characteristics of Endo-polygalacturonase on Alginate Beads

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ROZIE, H., SOMERS, W., BONTE, A., VISSER, J., VAN'T RIET, K., AND ROMBOUTS, F. M. Adsorption Characteristics of Endo-polygalacturonase on Alginate Beads. *Biotechol. Appl. Biochem.* **10,** 346–358 (1988).

The adsorption of endo-polygalacturonase (endo-PG) from a commercial enzyme preparation (Rapidase) to calcium alginate beads was studied. Approximately 75% of the polygalacturonase activity from Rapidase can be adsorbed at pH 4.4 by calcium alginate beads as well as by crosslinked sodium alginate powder. Equilibrium experiments were conducted to determine a parameter (k) that represents the degree of interaction between endo-PG and the adsorbent. This parameter can be influenced by a change in pH and ionic strength of the adsorbate. At pH 3.8 the degree of interaction is 20 times larger than that at pH 4.2. There is optimal adsorption when the ionic strength is lowered, although a small amount of $CaCl_2$ is required to keep the calcium alginate beads stable. Despite the resemblance in structure between L-guluronate blocks and polygalacturonate, it was shown that a decrease in k occurred when the alginate, used for the preparation of the beads, contained a large amount of guluronic acid. There is yet no evidence that L-guluronic blocks in the alginate chain are responsible for the large affinity of endo-PG to this adsorbent. The influence of the pH and the ionic strength and the lack of inhibition on endo-PG activity by sodium alginate are compatible with ionic interactions between endo-PG and the alginate chains.

Affinity chromatography is a well-known procedure for the purification of pectolytic enzymes such as pectin (methyl)esterase (1), pectate lyase (2), and endo-polygalacturonases (endo-PG).[1] The latter enzyme was purified on matrices derived from the natural substrate, pectate crosslinked by epichlorohydrin (3), or by using Separon, a poly(hydroxyethylmethacrylate) carrier modified by *O*-glycosidic coupling of trigalactosiduronic acid, as a ligand (4).

A disadvantage of crosslinked pectate was its slight biodegradation by the pectolytic enzyme mixture, which was not the case if crosslinked alginate, a modified substrate analog with affinity for endo-PG, was used instead (5).

[1] Abbreviations used: endo-PG, endo-polygalacturonase; M/G, mannuronic acid/guluronic acid ratio. Symbols used: K_a, adsorption equilibrium constant (liters/mol); K_c, moles of binding sites per gram of adsorbent; k, parameter for the degree of interaction (ml/mg); P_0, enzyme activity in solution before the addition of alginate beads (U/ml); P, enzyme activity of supernatant after the addition of alginate beads (U/ml); P_{ad}, enzyme activity bound on the adsorbent (U/ml); P_m, maximal fraction of P_0 bound on the adsorbent; P_{adm}, maximal adsorbed enzyme activity, the maximal value that is obtained for P_{ad} as P_0 is increased until P_{ad} does not change (U/ml); A, concentration of the alginate beads (mg/ml); B, enzyme activity per mmol protein (U/mmol), a constant value used in the experiment with partially purified enzyme; for endo-polygalacturonase this value is 1.05×10^8.

Alginate, industrially extracted from brown algae, is composed of varying and sometimes alternating sequences of L-guluronic and D-mannuronic acids. The glycosidic linkages between the L-guluronic acid residues lead to a diaxial orientation similar to that in pectate and equally suitable for an egg box type of chain association with calcium ions (6).

The crosslinking procedures described for pectate and alginate (3, 5) do not result in particles with a homogeneous size distribution. An alternative to crosslinking is the production of calcium alginate beads. The advantage of such beads is that they may be used in a fluidized bed reactor. In order to study the possibilities of using this system for the isolation of endo-PG on a technical scale, the parameters that affect the adsorption of the enzyme to the beads were investigated. The adsorption of endo-PG to alginate beads is described in terms of velocity and equilibrium as a function of pH and ionic strength.

Rexová-Benková (7) showed that the active site was involved in the selective binding of endo-PG to crosslinked pectate. The use of oligo-D-galactosiduronic acids of different degrees of polymerization as immobilized ligands confirmed that they functioned in a biospecific way (4). In this paper the question is also raised whether the same is true for binding of endo-PG to alginate beads which due to the polyelectrolyte character of the matrix may also have nonspecific ionic interactions with the enzyme.

MATERIALS AND METHODS

Alginate beads were produced from four different kinds of sodium alginate, namely, Manucol DM, extracted from *Ascophyllum nodosum*, and Manugel DMB, extracted from *Laminaria hyperborea* (stipes), both produced by Alginate Industries London, and two alginates extracted from a *Laminaria* sp. and *Laminaria cloustoni* respectively, obtained from Unipectine Paris.

The sodium pectate used to determine polygalacturonase activity was obtained from Sigma. The content of polygalacturonic acid is 85–90%.

The endo-PG solution was Rapidase C-80 (Batch 5031/5032 F1), a crude enzyme solution from *Aspergillus niger* produced by Gist brocades, Seclin, France.

Preparation of alginate beads. Beads were prepared by dropping 50 ml of 2% sodium alginate through a dropping funnel into a 100-ml 0.2 M $CaCl_2$ solution with continuously stirring. The beads obtained were stirred for 24 h in 0.2 M $CaCl_2$ with one replacement of the solution by a 0.2 M $CaCl_2$ solution. The beads were stored in a 0.03 M $CaCl_2$ solution at 4°C.

Large amounts of beads were prepared by making use of a resonance nozzle; 20 liters of alginate can be processed by this technique in 1 h (8). The bead diameter (1.50 ± 0.06 mm) was determined by using a binocular microscope (Zeiss-Ikon) with a measuring device.

Preparation of crosslinked alginate. Crosslinked alginate was prepared according to (1), by adding 17 ml of epichlorohydrin and 43 ml of 5 M sodium hydroxide to a suspension of 25 g of powdered sodium alginate in 100 ml of ethanol. The reaction mixture was shaken in a rotary incubator (175 rpm) for 4 h at 45°C and then neutralized with 7% acetic acid. The crosslinked alginate was isolated by filtration and successively washed with ethanol, ethanol/water (1/1), and water. The product was thoroughly washed again with ethanol/water (1/1) and ethanol after which the modified alginate was air-dried; the yield was 31 g.

Partial purification of endo-polygalacturonase from Rapidase C-80. The cross-linked alginate powder was suspended in 0.1 M sodium acetate buffer (pH 4.4) and packed in a chromatography column (28 × 180 mm). A Rapidase solution (500 ml, 35 U/ml) in the same buffer was loaded onto this column (flow, ca. 1.4 ml/min). Washing was performed with 500 ml of buffer (pH 4.4) after which the purified enzyme was eluted with 1 M sodium chloride in the same buffer. The eluted fractions were dialyzed against 0.1 M sodium acetate buffer (pH 4.2).

The enzymes in the partially purified enzyme solution were separated on a Mono-Q anion exchanger (FPLC apparatus, Pharmacia). The chromatography procedure was carried out using a 20 mM piperazine buffer (pH 6.0) with a gradient of 0–0.5 M sodium chloride and a flow of 0.5 ml/min.

Determination of enzyme activity. Polygalacturonase activity was determined with a modified ferricyanide test (9). The reaction mixture contained 2.0 ml of 0.25% sodium pectate in 0.1 M sodium acetate buffer (pH 4.2) and 20 μl of enzyme solution (0–17 U/ml). The mixture was incubated for 15 min at 30°C after which 150 μl was taken and added to 1 ml of 1% sodium carbonate. Next, 4 ml of a colored freshly prepared mixture (1:1) of a cyanide solution (0.25% KCN, 1% Na_2CO_3) and a ferricyanide solution (0.08% $K_3Fe(CN)_6$, 1% Na_2CO_3) was added. After standing for 20 min at room temperature, the reaction mixture (5.15 ml) was immersed in a boiling-water bath for 10 min and then immediately cooled with ice. Discoloration was measured spectrophotometrically at 420 nm after 1 h. The absorbance changes were interpreted in terms of reducing sugars by means of a standard graph for D-galacturonic acid. One unit (U) was defined as the amount of enzyme which released 1 μmol of reducing groups per minute.

Enzyme activities were also measured, using an autoanalyzer (Skalar, Breda, The Netherlands) by way of the neocuproin test (10). The peaks recorded were related to the unit defined by means of a standard graph for the Rapidase solution.

Determination of protein content. Proteins were precipitated by trichloroacetic acid in the presence of sodium deoxycholate (11). Succeeding protein determination with microbiuret reagent was performed spectrophotometrically at 330 nm (12).

End product analysis. To determine the action pattern of the enzyme in partially purified Rapidase, approximately 20 mU of enzyme from the fractions was incubated for 24 h at room temperature in 4 ml of 0.1 M sodium acetate buffer (pH 4.2) with 10 mg of sodium pectate. The breakdown products were determined by HPLC on an Aminex 42H experimental column (Bio-Rad, 30.0 × 7.8 mm); 25 μl of the sample was injected, and eluted with 0.01 M H_2SO_4 (flow, 0.6 ml/min) at 30°C.

Velocity of adsorption. Analyses were carried out under different experimental conditions. The rate of adsorption was measured as a function of the amount of alginate, ionic strength, and the pH.

Typically, 50 ml of an enzyme solution (17 U/ml) in sodium acetate buffer of defined pH (3.3–5.0) and ionic strength (NaCl concentration, 0–100 mM) was incubated under continuous stirring with an appropriate amount of alginate beads (50–200 mg/ml). Samples (1 ml) of the supernatant were taken in a time span of 1 h to be assayed for endo-PG activity. Sampling caused a reduction in volume (10% after 1 h). Corrections were not made as this did not change the data significantly.

The difference between the enzyme activity of the supernatant and the activity before the addition of alginate beads represented the amount of the enzyme bound.

Adsorption at equilibrium. The amount of alginate (1–40 mg/ml) was varied in a series of five batches to determine the degree of adsorption at equilibrium under certain conditions as a function of the composition of the alginate, the ionic strength (NaCl concentration, 0–100 mM; $CaCl_2$ concentration, 5–40 mM), and the pH (3.8–4.4). The activity of the enzyme bound at equilibrium was determined by the difference in enzyme activity of the initial enzyme solution and the activity in the sample taken after 2 days. A Rapidase C-80 solution and a partially purified enzyme solution were used in these experiments.

To determine more significant values from a physicochemical point of view, the initial enzyme concentration was also varied (10–45 U/ml) in a series of experiments keeping the amount of adsorbent constant (2 mg/ml).

Inhibition of enzyme activity by alginate. Polygalacturonase activities were measured at various concentrations of sodium pectate (0.02–0.1 mg/ml) in the presence or absence of sodium alginate (2 mg/ml) in 50 ml of 0.1 M sodium acetate buffer, pH 4.2. The enzyme concentration was 0.3 U/ml. The mixtures were incubated under stirring at 30°C for 15 min and at 2-min intervals, samples were taken and analyzed for reducing sugars.

RESULTS

Partial Purification of Endo-polygalacturonase from Rapidase

Commercial pectolytic enzyme preparations such as Rapidase C-80 usually contain several enzymes with polygalacturonase activity. H. C. M. Kester, H. Rozie, and J. Visser (unpublished results) identified at least four different endo- and two exo-polygalacturonases in Rapidase C-80 by purification and characterization. In order to analyze which of these enzymes binds to the alginate matrix, column chromatography using crosslinked alginate was applied. Rapidase C-80 was partially purified as described under Materials and Methods.

The fractions eluted with 1 M NaCl yielded a total of 75% of the original endo-PG activity and only 30% of the original protein content. Fast protein liquid chromatography with the purified enzyme solution showed that it contained mainly an enzyme with endo-PG activity and at least three other proteins, two of which also showed enzyme activity (Fig. 1). A molecular weight of 38,000 was determined by polyacrylamide gel electrophoresis for the major endo-PG in the purified Rapidase we used. Determination of the enzyme activity and the protein content of the FPLC fractions yielded the specific activities of the enzymes in this stage of purification (Table 1).

Aliquots of these enzyme fractions were incubated with a sodium pectate solution, and the end products were analyzed after complete hydrolysis. The endo-enzyme digest revealed three main products: the monomer, the dimer, and the trimer of D-galacturonic acid. The products of hydrolysis with the other enzyme was D-galacturonic acid only, showing that the enzyme was most probably an exo-enzyme.

The partially purified endo-PG, in which a single enzyme accounted for 91% of the polygalacturonase activity, was judged to be a suitable adsorbate in the adsorption studies.

An Equilibrium Model for Adsorption and Description of the Parameters that Affect Binding

Due to irregularities in the structure and size of the particles which arise from crosslinking alginate powder, we have prepared alginate beads to study the adsorption

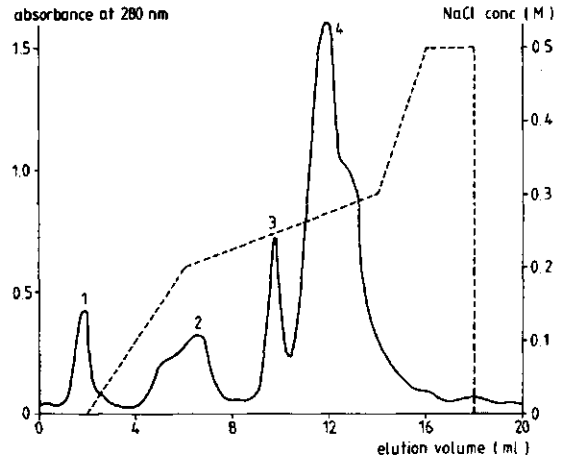

FIG. 1. FPLC elution profile of partially purified Rapidase. Peak numbers correspond to the enzymes listed in Table I.

process quantitatively. The adsorption of endo-PG to alginate beads depends on the adsorbent and the adsorbate. The factors that affect the adsorption are the size and the alginate content of the beads, investigated by W. Somers (unpublished results), as well as the kind of sodium alginate used to prepare the beads. The mannuronic acid/guluronic acid (M/G) ratio in the sodium alginate can influence the consistency of the beads and with that, or thereby, the adsorption of endo-PG to the adsorbent.

The ionic strength and the pH of the adsorbate are parameters that affect the adsorption. From the results of previous adsorption–desorption studies (5, 7) with crosslinked alginate and crosslinked pectate, less adsorption was expected with increasing ionic strength. A pH value of 4.2 was chosen for the adsorbate in the initial experiments. This is also the optimal pH value for pectate hydrolysis (13) and the often used value in other endo-PG studies (5, 7). Since calcium alginate beads must be stabilized by an amount of $CaCl_2$ in the adsorbate (minimally 5 mM), the influence of the $CaCl_2$ concentration on the adsorption reaction was also investigated.

Determination of the adsorption equilibrium constant K_a (liters/mol) and the moles of binding sites per gram of alginate beads (K_c) can be accomplished in a system

TABLE I

Composition of the Alginate Adsorbed Fraction of Rapidase

Protein	*Molecular weight*	*Protein (%)*	*Enzyme activity (%)*	*Specific activity (U/mg)*[a]
1 Endo-PG	n.d.	4	3	53
2 Exo-PG	95,000	15	7	89
3 Unknown	40,000	7	0	0
4 Endo-PG	38.000	68	91	580[b]

[a] The highest enzyme activity/protein ratio found in the FPLC fractions.

[b] Further purification of endo-PG (H. Kester, H. Rozie, and J. Visser, unpublished results) resulted in a specific activity of 2750 U/mg.

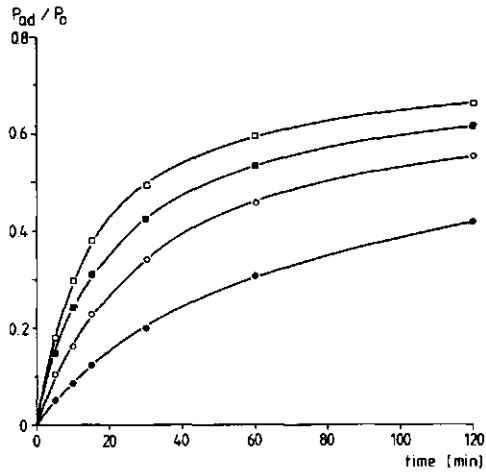

FIG. 2. Velocity of the adsorption of enzyme activity by alginate beads in 0.1 M sodium acetate buffer (pH 4.2), 5 mM $CaCl_2$, P_0 = 17 U/ml. ●, 50 mg/ml; ○, 100 mg/ml; ■, 150 mg/ml; □, 200 mg/ml alginate beads.

with increasing concentrations of the adsorbent, whereas the adsorbate remains the same. It can be shown that there is a relation between the adsorbed enzyme fraction and the amount of alginate beads added (14):

$$P_0/P_{ad} = 1/K_aK_c * 1/A + 1. \qquad [1]$$

A straight line should be found when the reciprocal of the adsorbed enzyme activity is plotted against the reciprocal of the amount of alginate beads, according to the method of Lineweaver and Burk (15). The reciprocal of the slope is a parameter which describes the degree of interaction between enzyme and adsorbent. Since K_a and K_c are determined as a product in this case (see Eq. [1]), another experiment in which the initial enzyme concentration is varied must be conducted in order to determine K_a separately. The relation between the amount of enzyme activity adsorbed and the amount of enzyme activity that remains in solution is (14)

$$1/P_{ad} = B/K_aP_{adm} * 1/P + 1/P_{adm}. \qquad [2]$$

When $1/P_{ad}$ vs $1/P$ is plotted, again a straight line should be found. The intercept at the ordinate is now equal to the reciprocal of the amount of enzyme that can be adsorbed by the amount of alginate beads used. The reciprocal of the slope is again a parameter for the degree of association from which K_a can be determined.

Adsorption of Crude Rapidase

To investigate the validity of the relations described, we used alginate beads prepared from Manucol DM in an adsorption experiment in which only the amount of alginate beads was varied. Enzyme activity in Rapidase was adsorbed by the beads as shown in Fig. 2. After 2 h equilibrium was not reached. With a small amount of alginate beads a reaction time of 2 days is required to reach equilibrium.

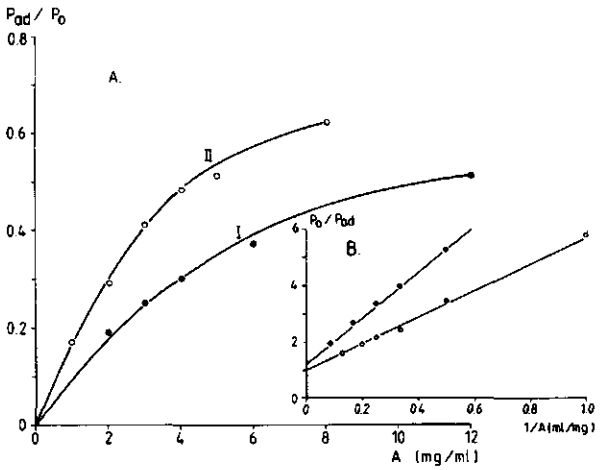

FIG. 3. The fraction of adsorbed enzyme activity from Rapidase (I) P_0 = 28 U/ml and partially purified Rapidase (II) P_0 = 18 U/ml at equilibrium, varying the amount of adsorbent in 0.1 M sodium acetate buffer (pH 4.2), 5 mM $CaCl_2$.

Determination of the enzyme fraction bound at equilibrium when different amounts of alginate beads are used revealed that the values are almost equal to one another (70–80%). Decreasing the amount of alginate reveals more distinction between these values. The measurements yielded a hyperbolic curve when the fraction of enzyme bound was plotted versus the amount of alginate beads and thus a linear relationship was found when the reciprocals were plotted (Fig. 3).

However, the intercept at the ordinate is not equal to 1, indicating that a fraction of the initial enzyme activity does not bind to the adsorbent. The intercept of the line is now equal to the reciprocal of the maximal fraction of enzyme activity bound (P_m). In this case the reciprocal of the slope (k = 0.125 ml/mg) cannot be equal to K_aK_c.

Adsorption of Purified Rapidase

The equilibrium experiment was repeated with partially purified polygalacturonase which represents the enzyme fraction in Rapidase C-80 that binds to alginate (Fig. 3). The intercept of the straight line is almost equal to 1, confirming that all the enzyme activity present in the purified enzyme solution can be adsorbed by the alginate beads. The reciprocal of the slope, k = 0.22 ml/mg, is now equal to K_aK_c.

Varying the initial enzyme concentrations, instead of the amount of alginate, yields a hyperbolic Langmuir-type plot (Fig. 4). When the reciprocal of the adsorbed enzyme activity was plotted versus the reciprocal of the nonadsorbed enzyme activity, a straight line was found. The equilibrium adsorption constant calculated from these data was found to be 2.1×10^6 liters/mol. The value of P_{adm} was converted from 25 U/ml to 1.2×10^{-7} mol of binding sites per gram of adsorbent. The determined K_a used with the data of Fig. 3 (partially purified enzyme solution) yields 1.0×10^{-7} mol/g for K_c.

A Langmuir-type plot was also found when Rapidase was used in this experiment instead of partially purified Rapidase. The data as obtained in Fig. 3 (Rapidase) were

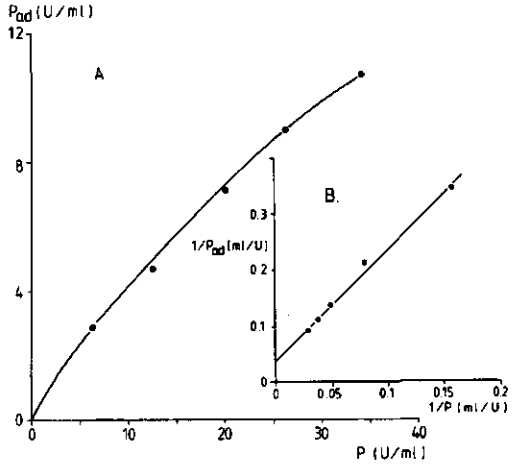

FIG. 4. The adsorption of enzyme activity from partially purified Rapidase at equilibrium, varying the initial enzyme concentration in 0.1 M sodium acetate buffer (pH 4.2), 5 mM $CaCl_2$, A = 2 mg/ml.

now corrected for the nonadsorbed fraction of enzyme activity in Rapidase. In this case we found for K_a 9.2×10^6 liters/mol and for K_c 0.37×10^{-7} mol/g. The decrease in K_c and the increase in K_a when Rapidase is used instead of the partially purified enzyme solution are inexplicable as yet.

Although the physical significance that can be attributed to K_a and K_c remains obscure due to the unbound fraction, the product (k) is still a useful parameter for defining the degree of interaction between enzyme and adsorbent. This is of value because the direct determination of K_a can be accomplished only if P_{adm} can be determined accurately and that is not possible if the degree of association between enzyme and adsorbent increases. Therefore we have chosen to determine k when comparing different adsorption conditions.

Effects of the Chemical Composition of Alginate

Since alginate isolated from different sources varies in mannuronic/guluronic acid ratio, the binding properties are likely to be influenced by the molecular composition of the polysaccharides. To investigate the relevance of the kind of alginate used to prepare alginate beads, the parameter k was determined for four different commercially available kinds of alginate. The results are summarized in Table II.

TABLE II

Adsorption of Polygalacturonase to Different Commercial Sodium Alginate Preparations

Sodium alginate	*M/G ratio*[a]	*Alginate content (%)*	*k (ml/mg)*
Manucol DM	1.85	5.1	0.125
Alginate from *Laminaria* sp.	1.15	5.1	0.110
Manugel DMB	0.45	4.1	0.050 (0.062)[b]
Alginate from *L. cloustoni*	0.35	4.1	0.058 (0.071)

[a] Value obtained from product specifications.

[b] Corrected for the alginate content of the beads compared with that of Manucol DM.

TABLE III

Determination of k at Different NaCl and $CaCl_2$ Concentrations
(0.1 M sodium acetate buffer, pH 4.2, P_0 = 17 U/ml)

$CaCl_2$ concentration (mM)	*NaCl concentration (mM)*	*Ionic strength (mM)*	*k (ml/mg)*
5	0	37	0.125
5	20	57	0.069
5	50	87	0.027
5	75	112	0.021
5	100	137	0.014
10	0	52	0.076
20	0	82	0.038
30	0	112	0.018
40	0	142	0.011

The sodium alginate Manucol DM appeared to be the best adsorbent for endo-polygalacturonase. The value of k for another alginate with a high M/G ratio derived from a *Laminaria* sp. was close to that of Manucol DM. These two kinds of alginate beads had an opaque appearance, in contrast with the other two which had a glassy appearance. Even when k was corrected for the difference in alginate content of the beads, it was obvious that the alginate beads with a low M/G ratio adsorbed less endo-PG.

The Influence of Ionic Strength

Desorption of endo-PG from crosslinked pectate and crosslinked alginate occurs with a large increase in ionic strength (3, 5). This implies that the binding process is also affected by the ionic strength. Measurements of the adsorption rate for Rapidase solutions showed a slower adsorption at larger NaCl concentrations. The determination of k confirmed that there is optimal adsorption when the ionic strength is lowered. The results are shown in Table III.

The presence of Ca^{2+} was required to keep the alginate beads stable. If the concentration of $CaCl_2$ increased from 5 up to 40 mM in a 0.1 M sodium acetate buffer (pH 4.2), less endo-PG was adsorbed. With regard to the ionic strength of the adsorbate, results similar to those with NaCl were found. It is shown that adsorption depends on the ionic strength of the adsorbate, regardless of the valency of the ions (Fig. 5). To prevent alginate leakage and to maintain an equal Ca^{2+} concentration in the enzyme solution, we preferred to use 5 mM $CaCl_2$ in all our experiments.

Effect of pH on Adsorption

Above pH 4.2 the adsorption of enzyme activity from Rapidase on alginate beads was expected to decrease, parallel with adsorption on crosslinked pectate and crosslinked alginate (5, 7). This was confirmed by our experiments. However, in contrast to the optimum pH of 4.2 measured for the adsorption of endo-PG on crosslinked pectate, we found that enzyme activity was more rapidly adsorbed by alginate beads

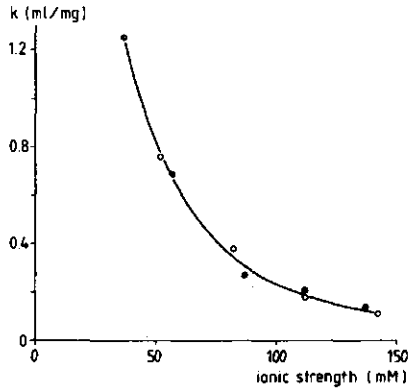

FIG. 5. The influence of ionic strength on parameter k. ●, Increasing NaCl concentration; ○, increasing $CaCl_2$ concentration.

below pH 4.0 (Fig. 6). The degree of interaction between enzyme and adsorbent as a function of pH was ascertained by determination of k at different pH values.

Figure 7 shows that when the pH is decreased, fewer alginate beads are needed to reach the same percentage of adsorption. Taking the reciprocal values of the curves as we did in Fig. 3, the parameter k at pH 3.8 is almost 20 times larger than that at pH 4.2. There were some difficulties in measuring the adsorption at equilibrium because the enzyme activity of the Rapidase solutions decreased with time below pH 3.8, probably due to denaturation. At pH 3.8 the stability of the Rapidase solution equals that of the enzyme at pH 4.2.

With regard to the large adsorption after 1 h at pH 3.3 and at pH 3.6, it is clear that there is no such optimal pH for the adsorption by calcium alginate beads, as found with crosslinked pectate. This is probably an indication that with alginate beads the interaction is of a different nature.

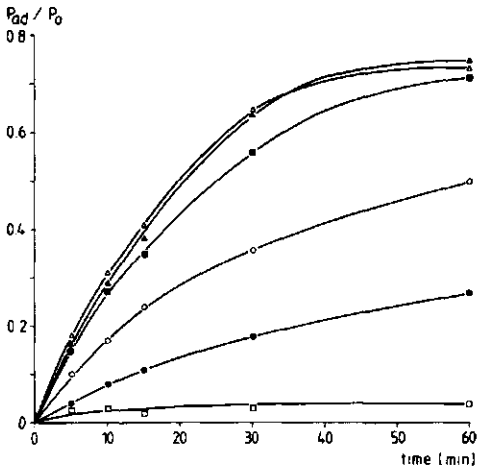

FIG. 6. The velocity of the adsorption of enzyme activity by alginate beads, varying the pH in 0.1 M sodium acetate buffer, 5 mM $CaCl_2$, P_0 = 17 U/ml, A = 50 mg/ml. ▲, pH 3.3; △, pH 3.6; ■, pH 3.8; ○, pH 4.0; ●, pH 4.2; □, pH 5.0.

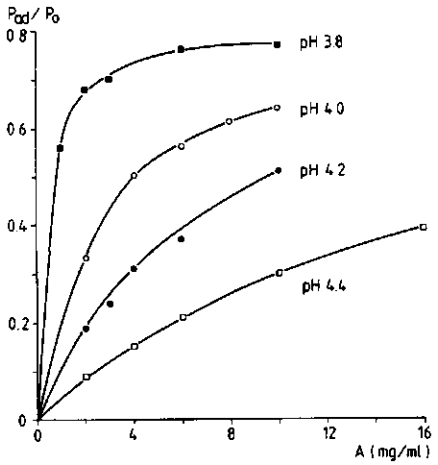

FIG. 7. The adsorption of enzyme activity from Rapidase at equilibrium, varying the amount of alginate beads at different pH values in 0.1 M sodium acetate buffer, 5 mM $CaCl_2$, P_0 = 30 U/ml. The calculated k values are for pH 3.8, k = 2.00; pH 4.0, k = 0.28; pH 4.2, k = 0.125; pH 4.4, k = 0.050.

Inhibition of Endo-polygalacturonase by Alginate

Rexová-Benková (7) showed that the biological activity of endo-PG was inhibited by crosslinked pectate, indicating that an active site-directed mechanism was involved in the selective binding of the enzyme. However, using an excess of alginate we were not able to find any inhibition of endo-PG activity, suggesting a kind of mechanism other than that found previously for the binding to crosslinked pectate.

DISCUSSION

It was shown that 75% of the polygalacturonase activity from Rapidase can be adsorbed by crosslinked alginate as well as by calcium alginate beads. The adsorbed enzyme activity consisted of three components, a high-molecular-weight endo-PG (5%), an exo-PG (4%), and the main endo-PG in Rapidase (91%). The enzymes present in the adsorbed fraction provide a breakdown pattern of the polymer substrate which is identical to that obtained with crude Rapidase. The application of an affinity adsorption step in the isolation process of technical enzymes thus leads to a preparation which is better defined and of higher purity. Whether this can be considered an advantage under all circumstances should be tested at the application level.

A suitable adsorbent for endo-PG can be produced if sodium alginate is crosslinked with epichlorohydrin. A change in the ratio epichlorohydrin/sodium alginate caused variations in the swelling and the adsorption capacity of the adsorbent. Calcium alginate beads are insoluble and stable in 5 mM $CaCl_2$ aqueous solutions. The preparation of crosslinked sodium alginate powder as well as that of calcium alginate beads was reproducible, leading to materials with constant adsorption capacities. When the beads were successively crosslinked with epichlorohydrin, the adsorption capacity decreased, whereas the stability of the beads was not significantly increased. Therefore the calcium alginate beads used for adsorption studies were made by complexation only.

Although calcium alginate beads must be stabilized with 5 mM $CaCl_2$ in contrast to the crosslinked alginate powder, they can be applied in fluidized bed adsorption. Mathematical adsorption models, treating both adsorption and penetration of the enzymes into the beads, were tested (W. Somers, unpublished results).

Equilibrium studies were carried out to investigate the adsorption capacity and the optimization of the adsorption conditions. Kinetic measurements were necessary to determine the time at which equilibrium is reached. A parameter k can be calculated by varying the amount of alginate beads in an experiment and measuring the adsorbed fraction of polygalacturonase at equilibrium. The parameter k is a product of K_a and K_c if the total amount of polygalacturonase activity can be adsorbed by the adsorbent. This is the case for partially purified Rapidase. In Rapidase we found a polygalacturonase fraction that was not adsorbed by calcium alginate beads and crosslinked sodium alginate. Thus for crude Rapidase the calculated parameter k does not represent K_a times K_c. When the data were corrected for the nonadsorbed enzyme activity in Rapidase, a lower K_c value and a higher K_a value were calculated, as was found for partially purified Rapidase. These differences can be explained by the nonadsorbed enzyme fraction in Rapidase. The adsorption of polygalacturonase from Rapidase to alginate beads at pH 4.2 in 0.1 M sodium acetate buffer revealed a value for k of 0.125 ml/mg. The value for k is 0.30 ml/mg if endo-PG is adsorbed by crosslinked pectate under the same conditions, as can be calculated from the data presented by Rexová-Benková (7). Comparison of these parameters, corrected for the dry weight of the adsorbents, shows an eightfold higher degree of interaction for the combination of endo-PG and alginate beads.

The degree of interaction on equilibrium, now represented by parameter k, can be influenced by a change in pH and ionic strength. At pH 3.8 the degree of interaction is 20 times larger than that at pH 4.2. An increase in the ionic strength by adding NaCl from 0 to 20 mM caused a twofold decrease in k. The addition of a divalent salt ($CaCl_2$) caused the same decrease in k as that expected from the increase in ionic strength (Fig. 5).

The composition of the alginate used for the preparation of the beads was also of importance. A larger content of L-guluronic acid (70 instead 35%) caused a twofold decrease in k. This is surprising since the L-guluronic acid blocks resemble polygalacturonate in structure, and one would therefore expect higher binding capacities of alginates having a low M/G ratio. On the other hand, one must keep in mind that the L-guluronic blocks have a larger affinity for Ca^{2+} than D-mannuronate (6, 16). Since Ca^{2+} ions are always present in the adsorbate, preferential binding of this ion to the guluronic acid residues will occur. The Ca^{2+} concentration ($\geq$5 mM) is such that this shields the L-guluronate blocks. This could be an explanation for the influence of the chemical composition of the alginate on parameter k. However, equilibrium experiments with crosslinked sodium alginate powder of different chemical compositions in calcium-free solutions showed similar results. Here, too, the sodium alginate with a smaller L-guluronic content showed a higher degree of interaction with polygalacturonases. Therefore, there is yet no evidence that L-guluronic blocks in the alginate chain are responsible for the large affinity of endo-PG to this adsorbent. The influence of the pH and ionic strength of the adsorbate on adsorption and the lack of inhibition on endo-PG activity by sodium alginate are compatible with ionic interactions between endo-PG and the alginate chains.

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Chapter 3

Rozie, H., Somers, W., van 't Riet, K., Rombouts, F.M., and Visser, J. (1991). Crosslinked potato starch as an affinity adsorbent for bacterial α-amylase. Carbohydr. Polym. 15, 349-365.

Crosslinked Potato Starch as an Affinity Adsorbent for Bacterial α-Amylase

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ABSTRACT

Crosslinked potato starch was prepared as an affinity adsorbent for bacterial α-amylase. To this end, reaction parameters for crosslinking in an ethanol/water solvent were investigated. The degree of crosslinking, and consequently the suitability of crosslinked starch as an adsorbent for α-amylase, changed by altering these parameters. An increase in the degree of crosslinking of the adsorbent caused lower affinity for bacterial α-amylase which resulted in an unfavourable decrease in adsorption capacity and a favourable decrease in the degradation of the adsorbent by the enzyme. 1 g of a suitable adsorbent for bacterial α-amylase, prepared with an epichlorohydrin/glucose monomer ratio of 0·65 (starch concentration 150 mg/ml, ethanol/water ratio 2·0, sodium hydroxide/epichlorohydrin ratio 1·0), can adsorb 9·8 mg of an α-amylase from B. licheniformis *at 4°C in 20 h.*

The equilibrium constant between bound and unbound α-amylase is dependent on the temperature. An effective desorption was possible by a shift to higher temperatures. Degradation values smaller than 0·1% were measured after an incubation of 1 h at 70°C in a desorption buffer with 20% glycerol.

It was concluded that coulombic interactions and hydrogen bonds are of no or little importance in enzyme adsorption. Van der Waals forces, which are responsible for the large temperature effect, are the main forces in the interaction between α-amylase and crosslinked starch.

INTRODUCTION

Affinity chromatography may ideally provide a quick one-step procedure for the purification of enzymes. This requires specific ligands which are often bound to Sepharose carriers. For amylases different systems have been worked out on an analytical scale. A proteinaceous α-amylase inhibitor from wheat kernel was used as a ligand for purification of insect α-amylase (Buonocore *et al.*, 1975). Wheat α-amylase was purified with immobilized β-cyclodextrin (Silvanovich & Hill, 1976) and immobilized glycogen (Tkachuk, 1975). Bacterial α-amylase however, does not bind to the β-cyclodextrin. No suitable ligand-carrier adsorbents are known for the purification of bacterial and fungal α-amylases although from an economic viewpoint the isolation of such amylases is quite important.

Another approach to the preparation of a suitable adsorbent for α-amylase is to modify its substrate. Starch granules, especially those of potato, are only slowly degraded by α-amylase due to the crystallinity of the granules (Banks & Greenwood, 1975). Modification, e.g. by crosslinking leaving the granules intact, will only lead to less affinity. However, after gelatinization of the granules, before or during the crosslinking reaction, the modified polymer chains become accessible to α-amylase. Starch can be crosslinked with epichlorohydrin, a bifunctional reagent that reacts with the hydroxyl groups of the glucose monomers, as if it were a di-epoxide. Two monomers are thus connected through a glyceryl bridge and a three-dimensional network will be formed. The required aqueous alkaline solution will lead also to side reactions, e.g. the formation of a monoglyceryl ether starch derivative which will not contribute to the formation of the network (Flodin, 1962). Thus, wheat starch was crosslinked and bacterial α-amylase was specifically adsorbed by this product (Weber *et al.*, 1976). In this case, loss of the amount of adsorbent occurred during its run for enzyme purification, although accurate degradation experiments were not carried out. Mateescu and Schell (1983) showed that the degradation of crosslinked amylose by bacterial α-amylase depends, as expected, on the degree of crosslinking.

In this paper the development of crosslinked potato starch, suitable for affinity chromatography of a commercial bacterial α-amylase, is described.

MATERIALS AND METHODS

Potato starch granules, extruded potato starch (Prejel EXP) and drum dried potato starch (Prejel WA4) were obtained from Avebe, Veendam,

The Netherlands. The soluble starch used to determine α-amylase activity was obtained from Merck, Darmstadt, FRG.

The enzyme solution in our investigations was Maxamyl (Batch MVA 1941), a heat stable bacterial α-amylase from *Bacillus licheniformis* produced by Gist brocades, Seclin, France. The amyloglucosidase from *Aspergillus niger*, used in degradation experiments, was obtained from Sigma, St Louis, USA.

Gelatinization and precipitation of potato starch

Gelatinization was carried out in an amylograph (Brabender, Duisburg, FRG). 450 ml of a 4% potato starch suspension in water was heated in 10 min to 50°C and then at a rate of 1·5°C/min up to 95°C. After 30 min the solution was cooled down at the same rate to 50°C. The slurry was added to 500 ml ethanol (96%) after which precipitation occurred. The supernatant was decanted and 300 ml ethanol was added. This procedure was repeated twice, first with ethanol and then with acetone. The powder was filtered and air-dried.

Determination of polymer size

The average polymer size of extruded and drum dried starch was determined by measuring the amount of reducing groups, with an autoanalyser (Skalar, Breda, The Netherlands) using an automated form of the neocuproin test (Stephens *et al.*, 1974). Maltose was used as a standard.

Crosslinking of potato starch

Gelatinized potato starch was suspended in a mixture of ethanol and water. The crosslinking reaction was carried out in a 100 ml flask. Then epichlorohydrin and 5 M sodium hydroxide were successively added. The ratio of ethanol/water (v/v) in the reaction mixture after addition of the 5 M sodium hydroxide solution varied between 0·75 and 3·0. The molar ratio of epichlorohydrin to glucose monomers (ECH/GM) varied between 0·50 and 1·50. The total volume of the liquid was about 45 ml. The reaction mixture was shaken in a rotary incubator for 4 h at 45°C and then neutralized with 7% acetic acid. The crosslinked starch was isolated by filtration and washed successively, with water (2×50 ml), ethanol (2×50 ml) and acetone (2×50 ml) after which the product was air-dried.

Determination of the degree of degradation

200 mg of crosslinked starch was suspended in 10 ml of a 1% Maxamyl solution in 0·1 M sodium acetate buffer. The reaction mixture was rotated for 20 h in a test-tube rotator at 40°C. Next, 200 μl of the supernatant was added to 790 μl 0·1 M sodium acetate buffer (pH 5·0) and 10 μl of a 1% (v/v) amyloglucosidase solution. This mixture was incubated for 20 h at 30°C. The amount of reducing sugars was determined as described above. D-glucose was chosen as a standard. In calculating the percentage degradation, the amount of starch assayed was corrected for the yield in the crosslinking reaction.

Determination of protein content

Proteins were precipitated with trichloroacetic acid in the presence of sodium deoxycholate (Bensadoun & Weinstein, 1976). Succeeding protein determinations with Lowry reagent were performed spectrophotometrically at 690 nm (Lowry *et al.*, 1951).

Determination of enzyme activity

α-Amylase activity was determined with a modified ferricyanide test (Rozie *et al.*, 1988). The reaction mixture contained 1·9 ml of 0·5% (w/v) soluble starch in 0·1 M sodium acetate buffer (pH 6·0) and 100 μl of an enzyme solution (0–0·5 U/ml). The mixture was incubated for 30 min at 30°C after which 200 μl was taken which was added to 800 μl of 1% sodium carbonate, precooled on ice. Next, 2 ml of a freshly prepared mixture (1:1) of a cyanide solution (0·25% KCN, 1% Na_2CO_3) and a ferricyanide solution (0·08% $K_3Fe(CN)_6$, 1% Na_2CO_3) was added. After 20 min at room temperature, the reaction mixture (3 ml) was immersed in a boiling-water bath for 10 min and then immediately cooled on ice. Discoloration was measured spectrophotometrically at 420 nm after 1 h. The absorbance changes were interpreted in terms of reducing sugars by means of a standard graph for maltose. One unit (U) was defined as the amount of enzyme which released 1 μmol of reducing groups per minute.

Enzyme activities were also measured with an autoanalyser (Skalar, Breda, The Netherlands) in which incubation of the enzyme with the substrate occurred. Determination of the reducing groups was carried out by the neocuproin test (Stephens *et al.*, 1974). The peaks recorded were related to the unit defined by means of a standard graph for the α-amylase solution.

Adsorption of α-amylase to crosslinked starch

10 ml 1% Maxamyl (50 U/ml) in 0·1 M sodium acetate buffer (pH 6·0) was incubated with 200 mg of crosslinked starch. The reaction mixture was rotated in a test-tube rotator for 20 h at 4°C. Samples of the supernatant were taken to determine the enzyme activity in solution and the degree of degradation of the matrix. The fraction of the original α-amylase activity present that could not be detected after incubation was supposed to be bound to the adsorbent.

Desorption of α-amylase from crosslinked starch

10 g of crosslinked starch (Table 1, ECH/GM = 0·65) was incubated with 500 ml 1% Maxamyl in 0·1 M sodium acetate buffer (pH 6·0) under

TABLE 1
Influence of the Amount of Epichlorohydrin on Yield and Properties of Crosslinked Starch

ECH/GM ratio	*Epichlorohydrin (mg/ml)*	*Degradation by α-amylase (%)*	*Adsorption of α-amylase (%)*	*Yield (%)*
(A)				
1·0	66·3	16·6	85	105·5
1·1	72·8	10·3	84	107·0
1·2	79·5	6·7	82	108·1
1·25	82·8	4·1	83	108·5
1·3	86·1	1·5	68	109·2
1·4	92·8	0·9	49	110·4
1·5	99·4	0·4	26	112·6
(B)				
0·8	52·5	12·4	80	106·3
1·0	65·7	3·9	85	108·3
1·2	78·8	0·3	44	110·9
(C)				
0·50	42·8	27·4	85	105·0
0·60	51·4	13·6	87	106·2
0·65	55·7	5·1	86	108·5
0·70	60·0	2·3	78	108·9
0·75	64·3	0·1	45	109·8

Reaction conditions: NaOH/ECH ratio is 1·0; reaction temperature is 45°C; reaction time is 240 min.
(A) Amount of starch is 115 mg/ml; E/W ratio is 3·65.
(B) Amount of starch is 115 mg/ml; E/W ratio is 2·70.
(C) Amount of starch is 150 mg/ml; E/W ratio is 2·00.

continuous stirring at 4°C. After 20 h the adsorbent was isolated by filtration and washed with cold water (100 ml, 4°C). A sample of the filtrate was taken to determine enzyme activity. Part of the wet solid (2%) was added to 10 ml desorption buffer. The reaction mixture was stirred in a test-tube rotator for 1 h at 70°C.

Desorption buffers were 0·1 M sodium acetate (pH 5·0–6·0), 0·1 M sodium succinate (pH 5·0–6·0) and 0·1 M sodium phosphate (pH 6·0–9·0). The ionic strength in the sodium acetate buffer (pH 6·0) was increased with sodium chloride up to 1 M. These buffers were also tested under adsorption conditions. Samples of the supernatant were taken after 1 h incubation at 70°C and after a 20 h incubation period at 4°C to determine the enzyme activity in solution and the degree of degradation of the matrix.

Column affinity chromatography

1 g of a crosslinked starch was swollen for 2 h at 4°C in 10 ml 0·1 M sodium acetate (pH 6·0). The adsorbent was used in a column with a diameter of 10 mm. 1 ml Maxamyl (2%, 100 U/ml) was loaded onto this column (flow, *c.* 0·3 ml/min). Washing was performed with 10 ml buffer at 4°C. Thereafter the column was put into a waterbath at 50°C. The adsorbed enzyme was eluted at 50°C using the same buffer, to which 20% glycerol was added.

RESULTS

A procedure to crosslink potato starch granules was published before by Kuniak and Marchessault (1972). In their study they used high sodium hydroxide concentrations which gelatinized the granules. They reported that a heterogeneous reaction took place when the sodium hydroxide concentration was too low, although the granules were considerably swollen. Therefore granules have to be gelatinized before crosslinking if the effects on the crosslinking reaction of sodium hydroxide concentration and of the solvent are to be investigated.

Potato starch granules were successively gelatinized and precipitated as described in Materials and Methods. Even when the gelatinization reaction was carefully standardized, crosslinked products from different gelatinizations showed different degrees of degradation and different affinity properties towards bacterial α-amylases. As shown in this paper, the crosslinking reaction of gelatinized potato starch itself is reproducible. Therefore, the different properties of the crosslinked starches to

α-amylase have to be ascribed to the poor reproducibility of the gelatinization and precipitation procedures in the laboratory.

For this reason, industrially prepared gelatinized potato starches were used. Reproducible specifications could now be given for the products obtained after modification with epichlorohydrin. Drum dried potato starch and extruded potato starch were crosslinked. Both affinity and stability towards bacterial α-amylase were measured for these matrices as described in Materials and Methods. Both adsorbents adsorbed α-amylase but the degradation of modified drum dried starch by α-amylase (5·1%) was lower than the degradation of modified extruded starch (11·7%) when they were crosslinked under the same reaction conditions (amount of starch 150 mg/ml; ECH/GM = 0·65; NaOH/ECH = 1; E/W = 2·00; 45°C; 240 min). As shown in earlier work on crosslinking of polysaccharides (Rombouts *et al.*, 1979), this could be due to the higher average polymer size of the drum dried potato starch (degree of polymerization 670) compared with extruded potato starch (degree of polymerization 310). We have chosen drum dried potato starch as a starting material to investigate in more detail those parameters which influence the crosslinking reaction.

Influence of reaction time and temperature on the crosslinking reaction

In Figs 1(A) and 1(B) it is shown that the yield of the crosslinked product, calculated on the basis of the amount of starch used in the reaction, and the degradation by α-amylase of the product are dependent on reaction time and temperature. In the degradation experiments, it was found that most adsorbents were solubilized by α-amylase. In spite of complete solubilization, no complete degradation was measured with amyloglucosidase, even if the product was only slightly crosslinked. Obviously, the oligomers solubilized by α-amylase cannot be degraded completely by amyloglucosidase. Incomplete degradation is probably due to glyceryl diethers and monoethers present in the oligomers.

Figure 1(B) shows that the yield of the product is an indication for the crosslinking of that product. All of the adsorbents with a yield below 109% solubilized completely with α-amylase in the degradation experiments, although the degree of degradation of the soluble dextrins with amyloglucosidase was less than 100%.

At temperatures of 25°C and 35°C prolonged reaction times are necessary to get sufficient crosslinking. For the reaction conditions chosen at a temperature of 45°C, a reaction time of 240 min is necessary to get a degree of crosslinking which is sufficiently high to minimize the degradation (approx. 5%) of the modified starch after treatment with

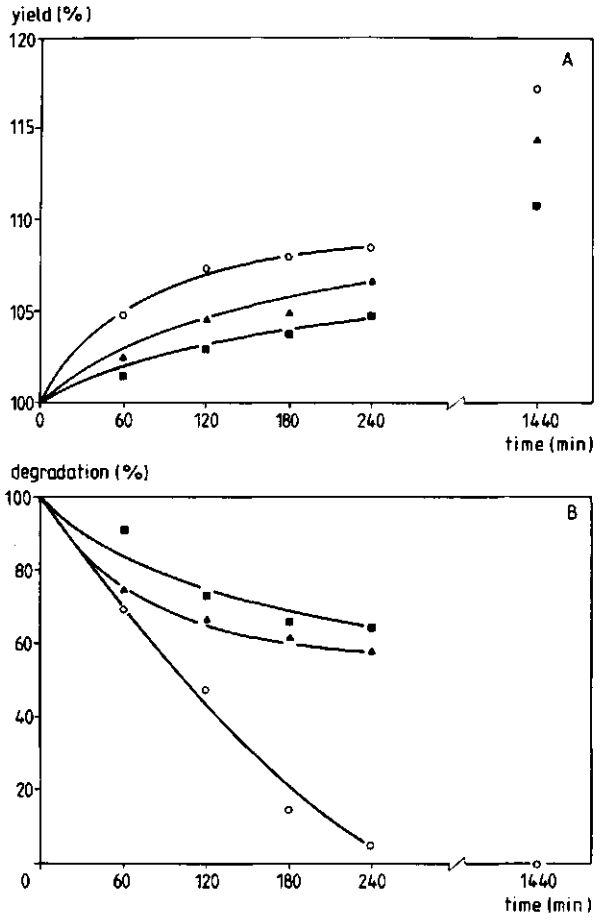

Fig. 1. The influence of reaction time and temperature on product yield of the crosslinking reaction (A) and on the degree of degradation of the product by α-amylase (B). Reaction conditions for crosslinking: amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0. Reaction temperature: ■, 25°C; ▲, 35°C; ○, 45°C. Degradation of crosslinked starch samples was tested by a 20 h incubation with 1% Maxamyl at 40°C as described in Materials and Methods.

α-amylase. These conditions were chosen to further optimize the solvent composition and the concentration of the other reactants in the crosslinking reaction.

Influence of the solvent on the crosslinking reaction

If water is the only solvent used in the crosslinking reaction and if the sodium hydroxide concentration is high enough, a homogeneous reaction will occur (Kuniak & Marchessault, 1972). The addition of ethanol makes starch insoluble which results in a heterogeneous reac-

tion. In this way a higher degree of crosslinking can be achieved. It was found that the ethanol/water ratio (E/W) is of importance for the degree of crosslinking (Fig. 2). Lowering E/W results in an increased yield and a decreased degree of degradation by α-amylase. The adsorption of α-amylase to the adsorbents is almost independent of the E/W ratio. If the E/W ratio falls below 2·0, the suspension starts to clot which makes preparation of a reproducible adsorbent difficult. For this reason other reaction parameters were studied at an E/W ratio of 2·0 or higher.

Influence of the amount of starch on the crosslinking reaction

Various amounts of starch were crosslinked in the same reaction volume, keeping the other parameters constant. The amounts of epichlorohydrin and of 5 M sodium hydroxide were varied proportionally with the amount of starch. In Fig. 3 it is shown that an increasing amount of starch leads to higher yields and less degradation. The adsorption of α-amylase is optimal between 130 and 150 mg of starch per ml and decreases rapidly with increasing amounts of starch, which makes the matrix practically resistant to enzymic attack. When low amounts of starch were used, the biodegradability of the adsorbents increased and with that the adsorption of α-amylase decreased.

It has to be kept in mind that together with the amount of starch the epichlorohydrin concentration has to increase in order to maintain the ECH/GM ratio. Thus the increasing degree of crosslinking could also be dependent on the absolute epichlorohydrin concentration.

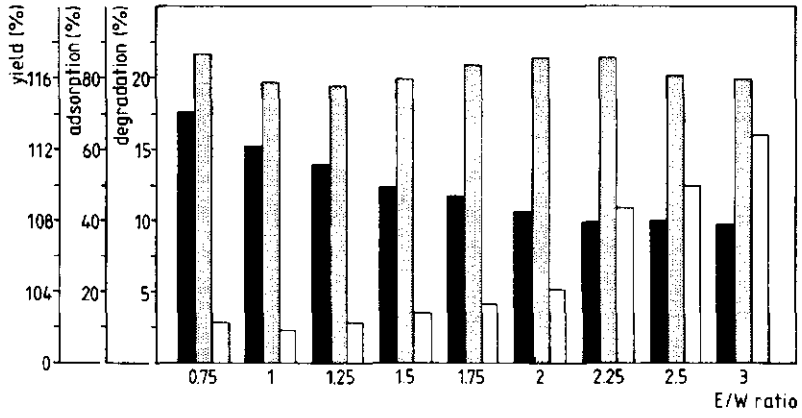

Fig. 2. Influence of the solvent composition in the crosslinking reaction on yield and properties of the adsorbent. Reaction conditions: amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; reaction temperature 45°C; reaction time 240 min. Solid bars, yield (%); hatched bars, adsorption of α-amylase (%); open bars, degradation by α-amylase (%).

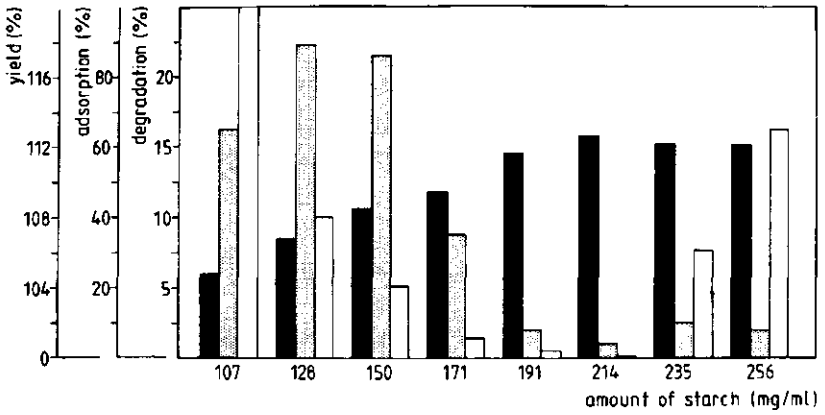

Fig. 3. Influence of the amount of starch used in the crosslinking reaction on yield and properties of the adsorbent. Reaction conditions: ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C; reaction time 240 min. Solid bars, yield (%); hatched bars, adsorption of α-amylase (%); open bars, degradation by α-amylase (%).

When the amount of starch exceeds 150 mg/ml, clotting occurs. This results in a lower reproducibility of the crosslinking reactor and thus leads to variation in the degree of crosslinking.

Influence of the concentration of epichlorohydrin and the ECH/GM ratio on the crosslinking reaction

Increasing the concentration of epichlorohydrin in the reaction mixture leads to higher yields and decreased biodegradability by α-amylase (Table 1(A)). From the yield of the crosslinked product it can be calculated that only a small part of the epichlorohydrin reacts with the gelatinized starch (15–20% if ECH/GM = 1·25). The amount of epichlorohydrin required to obtain an adsorbent that is stable to enzymic attack by α-amylase, can be lowered by increasing the amount of starch and/or decreasing the E/W ratio. A crosslinked starch with identical adsorption and stability properties with respect to α-amylase is obtained by a decrease of the E/W ratio from 3·65 to 2·70 and a decrease of the ECH/GM ratio from 1·25 to 1·0 (Tables 1(A) and 1(B)). Such an adsorbent can also be obtained with an ECH/GM ratio of 0·65. This can be achieved by a further decrease of E/W and an increase of the amount of starch (Table 1(C)). The ECH/GM range in which similar adsorbents are found, is smaller under these reaction conditions. As mentioned before, along with an increase in the amount of starch, an increase of the epichlorohydrin concentration occurs. ˇ th variation in the absolute

epichlorohydrin concentration and the changing ECH/GM ratio may have an effect on the degree of crosslinking. We could distinguish between these parameters by carrying out an experiment in which various amounts of starch (90–150 mg/ml) were crosslinked for 240 min at 45°C with a constant absolute epichlorohydrin concentration (55·7 mg/ml, NaOH/ECH = 1·0). The yields (108–109%) and the degrees of degradation (5–7%) and adsorption (80–87%) of the products prepared were about the same. Although there is an excess of epichlorohydrin in the reaction mixture with regard to the resulting yield, these results show that the degree of crosslinking depends not on the ECH/GM ratio or the amount of starch but on the absolute epichlorohydrin concentration in the reaction mixture.

Influence of the amount of sodium hydroxide (NaOH/ECH) on the crosslinking reaction

According to the reaction mechanism (Flodin, 1962) a NaOH/ECH ratio of 1 seems desirable. Kuniak and Marchessault (1972) obtained the highest degree of crosslinking with a ratio of 0·8 using water as the solvent. This value is in agreement with our results obtained with ethanol/water as the solvent. In the NaOH/ECH range of 0·6–1·0 the degree of crosslinking, based on degradation of the matrix by α-amylase, is almost equal, with an optimum at a NaOH/ECH ratio of 0·8 (Fig. 4).

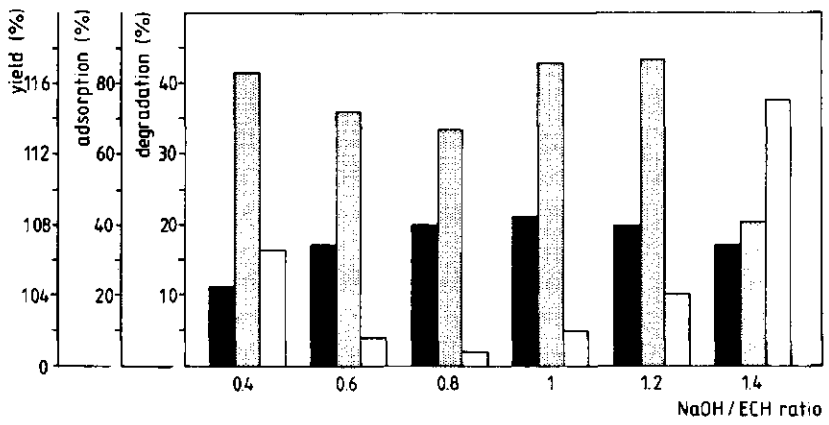

Fig. 4. Influence of the NaOH/ECH ratio in the crosslinking reaction on yield and properties of the adsorbent. Reaction conditions: amount of starch 150 mg/ml; ECH/GM ratio 0·65; E/W ratio 2·0; reaction temperature 45°C; reaction time 240 min. Solid bars, yield (%); hatched bars, adsorption of α-amylase (%); open bars, degradation by α-amylase (%).

Adsorption of α-amylase to crosslinked starch

The crosslinked starches were incubated for 20 h with 1% Maxamyl. The protein content of this enzyme solution was 375 μg/ml. Based on data from Table 1, it can be calculated that crosslinked potato starch can adsorb a considerable percentage of α-amylase activity at 4°C in 20 h (2150 U/g, 9·8 mg protein/g adsorbent).

A suitable adsorbent will have to be relatively stable to α-amylase. Crosslinked starches with adsorption values of 85% or more also have degradation values of 4% or higher. Potential adsorbents prepared in a solvent with an E/W ratio lower than 1·50 are exceptions to this rule. Clotting of the starch in the crosslinking reaction mixture may sometimes result in larger degradation values when the adsorbent is then incubated with α-amylase. Degradation values were all measured at 40°C. The best adsorption values were obtained at 4°C. The average percentage of degradation at 4°C was about half of the degradation measured at 40°C, both determined after 20 h.

Desorption of α-amylase from crosslinked starch

Competitive desorption with high maltose concentrations, up to 2 M, in the elution buffer was applied by Weber *et al.* (1976) to desorb α-amylase from crosslinked starches. From an economic point of view this is unrealistic and a cheaper method must be found. Our investigations showed that a low maltose concentration, 20 mg/ml (56 mM), in the desorption buffer at 4°C and 40°C or changes in pH and ionic strength did not increase α-amylose desorption. Indeed, with 1 M sodium chloride in the adsorption buffer an increase in adsorption from 26% up to 40% was measured (Table 1(A), ECH/GM = 1·5). An increase or decrease in pH of the elution buffer (5·0–9·0) or the use of other buffer salts like phosphate and succinate did not result in significantly different values for adsorption or desorption, although degradation varied (results not shown).

A temperature shift however, was effective in obtaining desorption. This method is attractive because of the temperature stability of the α-amylase. At 70°C desorption is complete within 10 min. The disadvantage of using high temperatures is that this leads to a rapid degradation of the matrix by α-amylase. However, degradation of the adsorbent can be minimized by the addition of 20% glycerol to the desorption buffer as is shown in Fig. 5. The addition of glycerol to the desorption buffer also has other advantages. There is a positive effect on the amount of α-amylase desorbed and on the stability of the heat stable α-amylase at high temperatures (Rozie *et al.*, unpublished results).

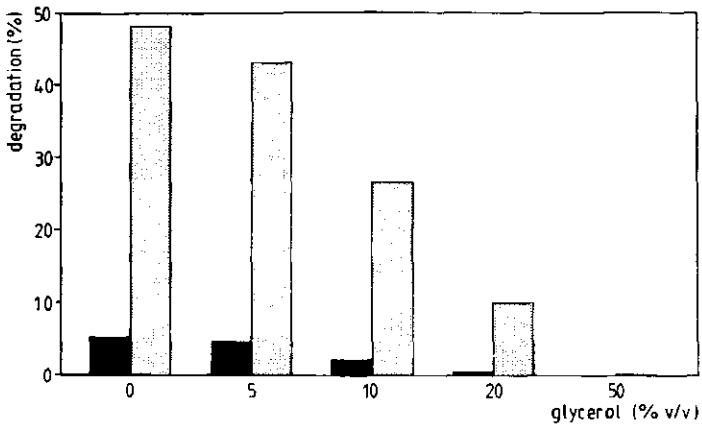

Fig. 5. The influence of glycerol on the degradation of crosslinked starch by α-amylase at 70°C. Reaction conditions for the synthesis of the adsorbents: amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C. Adsorbent A (solid bars), reaction time is 240 min; adsorbent B (hatched bars), reaction time is 120 min.

Application of crosslinked starch in column chromatography

Three crosslinked starches with different degrees of crosslinking were used to adsorb an α-amylase solution in a column as described in Materials and Methods (Fig. 6). If the degree of crosslinking is too high (adsorbent A), most of the enzyme elutes directly at 4°C. Only 10% of the enzyme activity adsorbs to the column and can be eluted at 50°C. The adsorbed fraction increases to 48% (Fig. 6(B)) and 85% (Fig. 6(C)) when the degree of crosslinking decreases. The biodegradability of these adsorbents was 0·0%, 0·1% and 5·1% respectively. As the commercial enzyme solution (Maxamyl) used in these experiments was already quite pure (*c.* 93%, Rozie *et al.*, unpublished results), these chromatographies did not result in an appreciable increase in specific activity. However, a good separation of α-amylase from contaminating phenolic compounds could be accomplished using a suitable crosslinked starch as an adsorbent.

DISCUSSION

The parameters of the crosslinking reaction of gelatinized potato starch with epichlorohydrin in an ethanol/water solvent have been investigated. Kuniak and Marchessault (1972) previously studied this crosslinking reaction, using starch granules in water. In their experiments the starch

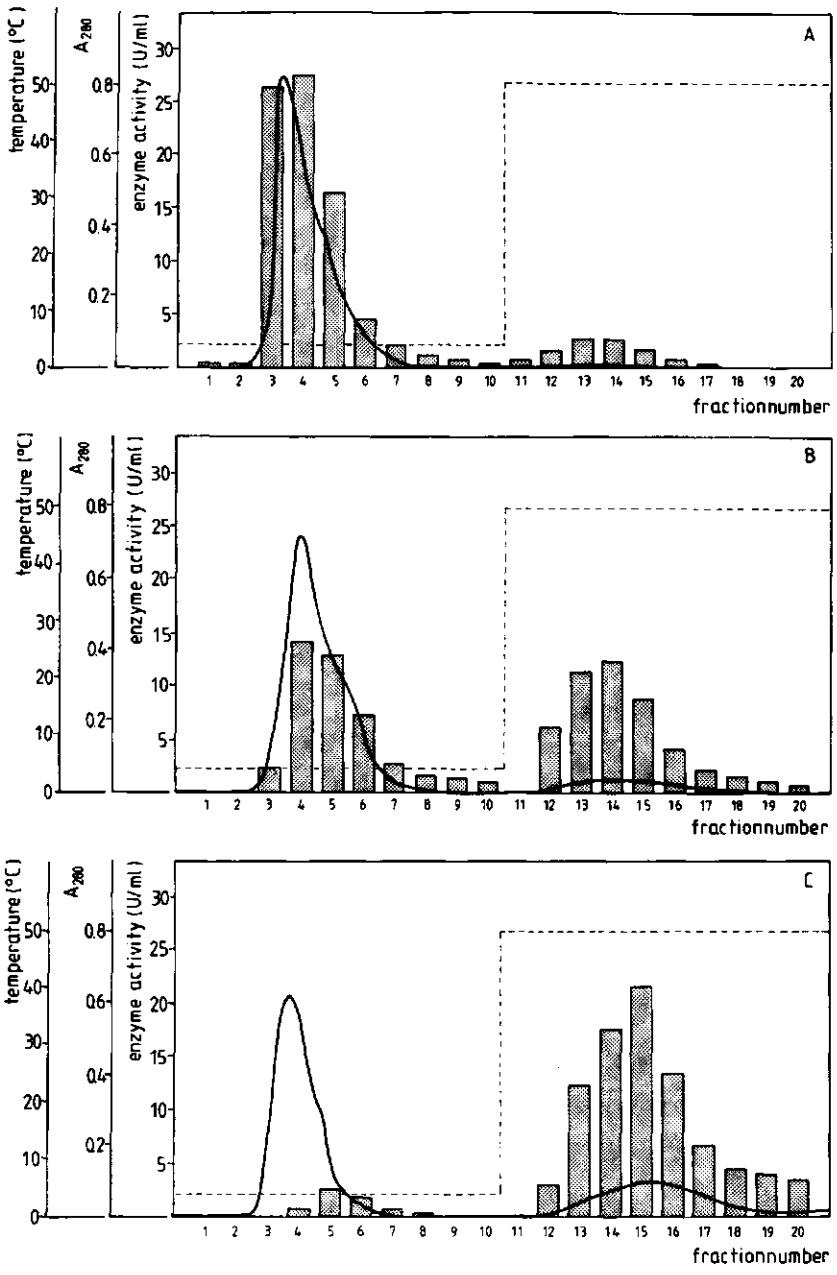

Fig. 6. Adsorption and desorption of bacterial α-amylase from three different cross-linked starches. Hatched bars, α-amylase activity (U/ml); ——, absorbance at 280 nm; – – –, temperature (°C). Reaction conditions for the synthesis of the adsorbents: (A) Amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C; reaction time 1440 min. (B) Amount of starch 150 mg/ml; ECH/GM ratio 0·75; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C; reaction time 240 min. (C) Amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C; reaction time 240 min.

granules were gelatinized by the high sodium hydroxide concentration in the reaction mixture. After a while the suspension solidified as a gel cake that was dispersed and washed with water and acetone. However, with such a product, which we prepared in preliminary experiments, we found a considerable amount of degradation by α-amylase. Another crosslinking procedure was introduced by Rombouts *et al.* (1979), who crosslinked polysaccharides in an ethanol/water solvent which yielded well defined products with adjustable swelling properties. Following these authors we also used ethanol/water solvents to crosslink potato starch. We had to use pre-gelatinized starch as a starting product since starch granules do not gelatinize that easily in ethanol/water mixtures.

The results of Kuniak & Marchessault (1972), with respect to the effects of the NaOH/ECH ratio, are confirmed by our own investigations. The effects of a variable ECH/GM ratio, however, are due to the variation of the absolute epichlorohydrin concentration. The degree of crosslinking changed by varying these parameters as detected indirectly with the degree of degradation of the different products by α-amylase. A decrease in degradation invariably correlated with decreasing adsorption values.

However, we found that a decrease in the E/W ratio of the solvent resulted in products with a lower degradation value, but with almost equal adsorption characteristics for bacterial α-amylase. With regard to minimizing biodegradability, it is favourable to choose an E/W ratio as low as possible. However, there is a limitation to this E/W value since gelatinized potato starch starts to clot if the E/W ratio falls below 2·0.

Decreasing degradation values in a series of crosslinked products can be explained by the higher degrees of substitution, that can be calculated from the higher yields obtained (Table 1). When the E/W ratio is varied, the crosslinked starch is equally accessible to α-amylase as can be concluded from the equal adsorption values, although the degree of substitution seems to change. A shift in reaction products, from the glyceryl diether bridges between the glucose monomers to the monoether derivative, may be an explanation for this phenomenon. A suitable adsorbent for bacterial α-amylase has to be reasonably stable with regard to this enzyme. Of the matrices prepared, we have particularly analysed a crosslinked starch (Table 1(C), ECH/GM = 0·65) which adsorbs 86% of a 1% Maxamyl solution in 20 h at 4°C (20 mg/ml adsorbent). 1·8% of the adsorbent is degraded under these conditions.

The adsorption velocity of this material has been further optimized (Somers *et al.*, unpublished results). Thus a matrix has been prepared which has a high adsorption capacity for α-amylase and which is slowly degraded by this enzyme whereas only an incubation time step of *c.* 10 min is required for adsorption.

The equilibrium constant between bound and unbound α-amylase is dependent on temperature. An effective desorption was possible by a temperature shift to higher values. Degradation values smaller than 0·1% were measured after an incubation of 1 h at 70°C in a desorption buffer with 20% glycerol. Dissociation did not occur with 1 M sodium chloride at low temperature (and no glycerol in the desorption buffer). Thus, coulombic interactions do not play an important role in the interaction (Van Oss *et al.*, 1986) so we must consider the role of van der Waals forces and/or hydrogen bonds. Hydrogen bonding becomes weaker with decreasing temperature and on the addition of chaotropic agents in the solvent. However, a decrease in temperature had a positive effect on the interaction between amylase and crosslinked starch, although the addition of a chaotropic agent, 200 mM guanidine hydrochloride, did result in a small decrease in adsorption of α-amylase (results not shown). Thus, hydrogen bonding seems to be of little importance and other interactions have to be responsible for the large temperature effect observed. Van der Waals forces are negatively influenced by an increase in temperature and a decrease in dielectric constant of the solvent (Van Oss *et al.*, 1986). Therefore, van der Waals forces play an important role in the interaction between α-amylase and crosslinked starch.

The application of crosslinked starch as an adsorbent for α-amylase on an analytical scale in column chromatography is shown in Fig. 6. The amount of adsorbed enzyme depends on the degree of crosslinking of the adsorbent. Batch experiments with long adsorption times showed, however, that the capacity of adsorbents B and C is considerably higher than one may assume from these column chromatography experiments, performed under non-equilibrium conditions. This will be covered in the next paper.

ACKNOWLEDGEMENT

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Chapter 4

Rozie, H., Somers, W., Bonte, A., van 't Riet, K., Visser, J., and Rombouts, F.M. (1991). Adsorption and desorption characteristics of bacterial α-amylases on cross-linked potato starch. Biotechnol. Appl. Biochem. 13(2), 181-95.

Adsorption and Desorption Characteristics of Bacterial α-Amylases on Cross-Linked Potato Starch

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ROZIE, H., SOMERS, W., BONTE, A., VAN'T RIET, K., VISSER, J., AND ROMBOUTS, F. M. Adsorption and Desorption Characteristics of Bacterial α-Amylases on Cross-Linked Potato Starch. *Biotechnol. Appl. Biochem.* **13**, 181–195 (1991).

The adsorption and desorption characteristics of two bacterial α-amylases (*Bacillus subtilis*, *Bacillus licheniformis*) on cross-linked potato starch were studied. The capacity of the adsorbent is dependent on the degree of cross-linking. A capacity of about 185 mg (*B. subtilis*) and 71 mg (*B. licheniformis*) protein per gram adsorbent can be realized. The adsorption constant (K_a) decreases with increasing temperature showing that association is caused by van der Waals forces. At 4°C a smaller K_a was measured for the enzyme from *B. subtilis* (0.53×10^5 liter/mol) than for the *B. licheniformis* enzyme (3.8×10^5 liter/mol). Comparison of the adsorption of the α-amylases to cross-linked starch with the activity of the enzymes on their natural substrate reveals that the velocity constant of the backward reaction of the enzyme–adsorbent complex increases strongly with increasing temperatures (*B. subtilis* α-amylase, $k_2(20°C)/k_2(4°C) \sim 30$). The adsorption velocity of α-amylases to freshly cross-linked starch is low due to the low accessibility of the adsorbent. This can easily be improved by enzymatic modification. Desorption can be accomplished by a raise in temperature. Glycerol (20%) is added to the desorption buffer to stabilize the enzymes and protect the adsorbent against enzymic attack. The optimal desorption temperature for the *B. subtilis* enzyme is 60°C. For the *B. licheniformis* enzyme this value is 70°C or even higher. Thus, bacterial α-amylases can be adsorbed and desorbed within short time spans (10 min) in sufficiently high amounts to make such an affinity purification process economically feasible.

Enzymes capable of hydrolyzing starch are numerous, and the properties of these enzymes are considerably different. Microbes are widely used for production of starch-degrading enzymes, which are used mainly in the food and beverage industry (1). The saccharide-converting enzymes have to be recovered from the process medium which is usually accomplished by removal of cells and a solid–liquid separation (2). Recovery of the enzymes by affinity separation would be an improvement as it may ideally provide a quick one-step procedure which leads to highly purified enzymes.

Such an affinity separation could be based on an enzyme–substrate interaction. Cross-linked starch was applied as an affinity adsorbent for a bacterial α-amylase on

an analytical scale (3). A scale-up of this process is possible if the following conditions are fulfilled:

(a) Enzyme–adsorbent complex formation should develop quickly.

(b) Desorption must take place in a desired rate.

(c) The adsorbent and the enzymes have to be stable during the adsorption–desorption process.

In recent studies (4) we found that a suitable cross-linked starch with a specific degree of cross-linking can be synthesized in different ways by altering the main parameters: the absolute epichlorohydrin concentration, the ethanol/water ratio, the NaOH/epichlorohydrin ratio, reaction time, and temperature. High specificity of the adsorption step was described. It was found that van der Waals forces are responsible for association between cross-linked starch and a bacterial α-amylase. Desorption could not be accomplished by a change in pH or ionic strength (4). Desorption occurred competitively with 2 M maltose (3) or by disruption of van der Waals bonds by an increase in temperature (4).

A disadvantage of this adsorbent was its slight biodegradation by the amylolytic enzyme mixture, although this could be reduced by the addition of glycerol. The low initial adsorption velocity was also an obstacle for the development of a large-scale purification process of bacterial α-amylases (4). For an economically competitive adsorption–desorption process, adsorption velocity and stability of adsorbent in the presence of the enzyme had to be improved. Therefore, the adsorption and desorption characteristics of two bacterial α-amylases on cross-linked potato starch have been investigated as discussed in this paper.

MATERIALS AND METHODS

Drum-dried potato starch (Prejel WA4) was obtained from Avebe (Veendam, The Netherlands).

The soluble starch used to determine α-amylase activity was obtained from Merck (Darmstadt, FRG).

The bacterial α-amylases in our investigations were Dexlo-CL and Maxamyl produced by Gist brocades (Seclin, France). According to the product sheet Dexlo-CL α-amylase originates from *Bacillus subtilis*.

Determination of Enzyme Activity

α-Amylase activity was determined with a modified ferricyanide test (4, 5). One unit was defined as the amount of enzyme which released 1 μmol of reducing groups per minute.

Enzyme activities were also measured with an autoanalyzer (Skalar, Breda, The Netherlands) in which incubation of the enzyme with the substrate occurred. Determination of the reducing groups was carried out by the neocuproin test (6). The peaks recorded were related to the unit defined by means of a standard graph for the α-amylase solution.

Determination of Protein Content

Proteins were precipitated by trichloroacetic acid in the presence of sodium deoxycholate (7). Subsequent protein determination with Lowry reagent was performed spectrophotometrically at 690 nm (8).

Action Patterns on Starch of Bacterial α-Amylases

To determine the action pattern of the α-amylases, 0.5 U of enzyme was added to 10 ml of 20 mM sodium acetate buffer (pH 6.0) which contained 10 mg/ml soluble starch (Merck). These mixtures were incubated under stirring at 30°C. Samples (1 ml) were taken in a time span of 4 h. From the product sheets of the commercial α-amylases it was known that the enzymes are strongly inhibited by heavy metals. Enzyme reaction was stopped by adding 0.5 ml of a 1 M lead(II) nitrate solution. Subsequently, the mixtures were immersed in a boiling water bath for 20 min. The breakdown products were determined by HPLC (SP 8000, Spectraphysics, San Jose, CA, U.S.A.) equipped with an Aminex-HPX-42A column (300 × 7.8 mm, Bio-Rad, Richmond, CA, U.S.A.) and a guard column (50 × 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 was operated at 85°C, the guard column at ambient temperature. Elution was carried out with water at a flow rate of 0.5 ml/min. Sugars were detected with an ERMA-ERC 7510 refractive index detector, thermostated at 40°C.

Kinetics of α-Amylase Activity

α-Amylase activities were measured at various concentrations of soluble starch from Merck (0.1–5 mg/ml) in 5 ml of 0.1 M sodium acetate buffer (pH 6.0). The enzyme concentration was 25 mU/ml for Maxamyl and for Dexlo α-amylase. The mixtures were incubated under stirring at various temperatures (4–40°C) for 1 h. With 10-min intervals, samples were taken and analyzed for reducing sugars. From Lineweaver–Burke plots the Michaelis–Menten constants and the maximum velocities of the α-amylases were calculated.

Preparation of Cross-Linked Starch

Cross-linked potato starch was prepared as described before (4). The cross-linking reaction was carried out in a 100-ml flask. Gelatinized potato starch (7 g) was suspended in an ethanol/water mixture (28.95 ml ethanol, 9.97 ml water). A 2.3-ml sample of epichlorohydrin and 5.62 ml 5 M sodium hydroxide were successively added. The molar ratio of epichlorohydrin to glucose monomers (ECH/GM)[2] was 0.65. The reaction mixture was shaken in a rotary incubator for 4 h at 45°C and then neutralized with 7% acetic acid. The cross-linked starch was isolated by filtration and washed, successively with water (2 × 50 ml), ethanol (2 × 50 ml), and acetone (2 × 50 ml), after which the product was air-dried (adsorbent X).

An adsorbent (Y) more stable toward α-amylase activity was prepared by increasing the amount of starch and the absolute epichlorohydrin concentration in the cross-linking reaction (4). Thus, 8 g of potato starch was suspended in 28.62 ml ethanol and 9.17 ml water. Next, 2.63 ml epichlorohydrin and 6.42 ml 5 M sodium hydroxide were successively added. Reaction conditions and washing procedure were kept the same.

[2] Abbreviations used: ECH, epichlorohydrin; GM, glucose monomers; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Purification of α-Amylase by Column Affinity Chromatography

A 1-g sample of a cross-linked starch (X) was swollen for 2 h at 4°C in 10 ml 0.1 M sodium acetate (pH 6.0). The adsorbent (about 5 ml) was used in a column with a diameter of 10 mm. The column was thermostated in a water bath. Water at 4°C was circulated from a cooling water bath (Haake F3K, Karlsruhe, FRG). Either 0.5 ml Maxamyl (50%, 1250 U) or 0.5 ml Dexlo (50%, 2500 U) was loaded onto this column (flow, ca. 0.3 ml/min). Washing was performed with 10 ml buffer at 4°C. Thereafter, the temperature was increased up to 50°C. The enzyme was eluted at 50°C using the same buffer, to which 20% glycerol had been added.

SDS–PAGE Electrophoresis and FPLC Patterns

Electrophoresis of crude and purified enzymes was carried out with the Phast-system apparatus (Pharmacia). Marker proteins were obtained from Bio-Rad (low MW standards). The gel plate was colored with Coomassie brilliant blue (PS Development Technique File No. 200).

The crude enzymes and the enzymes purified with cross-linked starch were separated on a Mono S cation exchanger (FPLC apparatus, Pharmacia). The chromatography procedure was carried out using a 50 mM sodium acetate buffer (pH 6.0) with a gradient of 0–0.5 M sodium chloride and a flow of 1 ml/min.

Thermostability of Bacterial α-Amylases

A 2% Dexlo and a 2% Maxamyl α-amylase solution in 0.1 M sodium acetate buffer were held at 60 and 70°C, respectively. Samples, taken in a time span of 2 h, were assayed for α-amylase activity. To study the influence of glycerol on thermostability of the enzyme the same experiment was carried out with 20% glycerol in the buffer solutions.

Adsorption at Equilibrium

The initial enzyme concentration was varied in a series of six batches to determine the degree of adsorption at equilibrium under defined conditions. The amount of enzyme bound at equilibrium was determined as the difference in enzyme activity of the initial enzyme solution and the activity in the sample taken from the supernatant after 48 h. Two kinds of cross-linked starch (as described above) as well as two kinds of α-amylase (Maxamyl, 25–100 U/ml, 0.5–2%, and Dexlo, 100–400 U/ml, 1–4%) were used in these experiments. The adsorbent concentration was 20 mg/ml. The suspensions (7.5 ml) were agitated at 4 and 20°C in a test tube rotator.

Improving the Adsorption Kinetics

A 7.5-ml sample of an α-amylase solution (100 U/ml) was incubated with 150 mg cross-linked starch (X) and agitated in a test tube rotator at two different temperatures (4 and 20°C). Samples of the supernatant (50 μl) were taken in a time span of 72 h to be assayed for α-amylase activity. Sampling caused a reduction in volume (about 5% after 72 h). Volume corrections were not made as this did not change the data significantly.

Velocity of Desorption

An 8-g sample of cross-linked starch (X) was suspended in 400 ml of a Maxamyl α-amylase (100 U/ml) or a Dexlo α-amylase solution (200 U/ml) in 100 mM sodium acetate buffer (pH 6.0). The suspensions were stirred magnetically at 4°C. After 2 days the adsorbents were isolated by filtration. The filtrate contained 26% of original Maxamyl and 44% of original Dexlo α-amylase activity. The wet adsorbents were washed once with 50 ml cold adsorption buffer. The washings contained about 0.5% for Maxamyl and 3% for Dexlo α-amylase of the original enzyme activity. The wet adsorbents were weighed and divided up in 40 portions (2.5% of the wet weight). These portions of enzyme-loaded adsorbent were used for desorption.

The desorption buffers used were 100 mM sodium acetate (pH 6.0) and the same buffer with 20% glycerol (v/v). The temperatures of the desorption buffers used were 40, 50, 60, and 70°C. Desorption times were 2, 10, and 30 min. After the desired time span, the suspended material was centrifuged as quickly as possible at room temperature (about 30 s). A sample of the supernatant was taken, cooled on ice, and analyzed for enzyme activity.

The Adsorbent in a Second Adsorption–Desorption Cycle

The Maxamyl α-amylase-loaded adsorbent described above was completely desorbed by conducting the desorption step twice for 10 min at 70°C with 20% glycerol in the desorption buffer. Subsequently, the adsorbent was washed with water, ethanol, and acetone. For Dexlo α-amylase the desorption temperature was 60°C. The air-dried products were reused in a second adsorption–desorption cycle.

RESULTS

Properties of Bacterial α-Amylases

Action patterns of bacterial α-amylases on starch. Degradation studies as described under Materials and Methods were carried out to determine which α-amylases are present in the commercial batches we used in our experiments. The results are shown in Fig. 1. The amount of starch oligomers produced by Dexlo α-amylase corresponds with the degradation pattern of the α-amylases from *B. subtilis* and *Bacillus amyloliquefaciens*. According to Robyt and French (9) α-amylase from *B. subtilis* has nine subsites with a cleavage point between the sixth and the seventh subsite from the nonreducing end. The asymmetric position of the cleavage point determines that G4 and G5 will not be produced in high amounts. Similar results were obtained by Thomas and Allen (10) with α-amylase from *B. amyloliquefaciens*. Although the results of Fig. 1 do not allow discrimination between these two sources of α-amylase, the results are in agreement with the product sheet from Gist brocades, which indicates *B. subtilis* as the enzyme source.

The degradation pattern of Maxamyl α-amylase corresponds with that of *Bacillus licheniformis* as described by Morgan and Priest (11) and Inglett (12). This enzyme leads initially to pentamer and larger products, later followed by production of pentamer, trimer, dimer, tetramer, and monomer products in descending order.

Kinetics of α-amylase. The Michaelis–Menten constants (K_m) and the corresponding maximum velocities (V_{max}) for Maxamyl and Dexlo α-amylases have been determined at several temperatures. The results are shown in Table I. The K_m values of

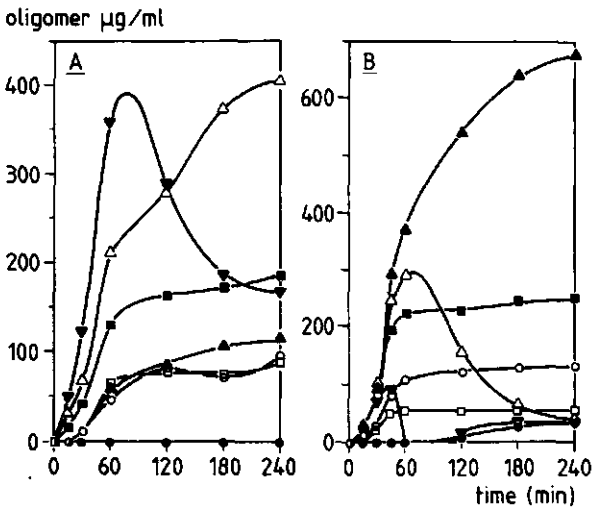

FIG. 1. The action patterns of two α-amylases, (A) Dexlo and (B) Maxamyl, on starch (●, monomer; ○, dimer; ■, trimer; □, tetramer; ▲, pentamer; △, hexamer; ▼, heptamer).

Maxamyl are lower than those of Dexlo α-amylase, showing that the initial reaction velocity of Maxamyl α-amylase reaches its optimal value at much lower substrate concentrations compared to Dexlo α-amylase. The value of V_{max}, thus the velocity of desintegration of the enzyme–substrate complex into the enzyme and its products, is of the same order of magnitude and increases with increasing temperature in both cases. K_m increases with increasing temperature for Maxamyl α-amylase and not at all for Dexlo α-amylase.

SDS–PAGE and FPLC patterns. The molecular weights of the α-amylases (Maxamyl, 60 kDa; Dexlo 64 kDa) have been determined with SDS–PAGE. SDS–PAGE of purified α-amylases and the commercial enzyme preparations showed that crude Maxamyl α-amylase is already quite pure compared to Dexlo α-amylase. Fast protein liquid chromatography (Fig. 2) showed that the purified Maxamyl α-amylase solu-

TABLE 1

Values of the Michaelis–Menten Constant and the Maximum Velocity for Maxamyl and Dexlo α-Amylases at Different Temperatures

	Maxamyl α-amylase		*Dexlo α-amylase*	
T (°C)	K_m (mg/ml)	V_{max} [(mmol/liter · min) × 10^{-5}]	K_m (mg/ml)	V_{max} [(mmol/liter · min) × 10^{-5}]
4	0.130	0.34	1.05	0.95
10	0.136	0.78	1.07	1.8
20	0.166	1.5	0.94	2.8
30	0.225	1.9	1.03	3.3
40	0.309	3.0	1.07	3.8

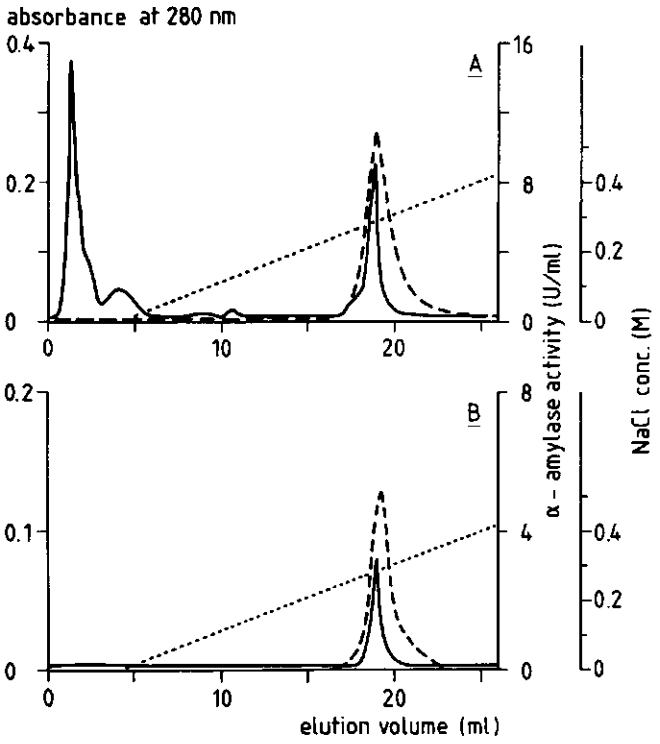

FIG. 2. FPLC elution profiles of crude (A) and purified (B) Maxamyl α-amylase (—, E280; ---, α-amylase activity).

tion is largely purified from contaminations present in the crude enzyme. With regard to the commercial product the specific activity (A_{sp}) for Maxamyl α-amylase increased from 192 to 220 U/mg in the FPLC fractions. Dexlo α-amylase could not be separated from contaminating proteins by ion-exchange FPLC. A_{sp} was 430 U/mg for purified Dexlo α-amylase compared with 279 U/mg for crude Dexlo α-amylase.

The stability of the α-amylases. According to the literature (13), most α-amylases from *B. subtilis* and *B. licheniformis* are stable in a pH range from 6 up to 11. Both enzymes are reported to have broad pH optima (around pH 6). Our results confirmed these data. Acetate buffer (pH 6.0) could be used in all adsorption and desorption experiments, as affinity of the enzyme to the adsorbent was hardly dependent on pH (4). Since temperature is an important parameter in the reaction of α-amylases with cross-linked starch, high-temperature stability is required. The α-amylase of *B. licheniformis* is reported to be a thermostable enzyme, used in applications at higher temperatures (14). We found that one-half of Maxamyl α-amylase activity is lost in ca. 95 min at 70°C. The half-life of Dexlo α-amylase at 60°C is ca. 120 min. Both enzymes could be stabilized by 20% glycerol (v/v) in solution. In that case, no loss of activity was measured within a time span of 2 h.

Adsorption Characteristics

Adsorption at equilibrium. A description of the adsorption reaction according to a simple equilibrium equation is valid if (a) we consider the reaction between α-amy-

TABLE II

Adsorption Equilibrium Constants and Adsorbent Capacities for Maxamyl and Dexlo α-Amylases

Enzyme	*Adsorbent*[a]	*T* (°C)	P_{adm}[b] (U/ml)	K_a[c] (liter/mol × 10^5)
Maxamyl	X	4	311 ± 50	3.8 ± 0.6
Maxamyl	X	20	247 ± 40	1.7 ± 0.3
Maxamyl	Y	4	107 ± 20	4.4 ± 0.8
Maxamyl	Y	20	186 ± 40	1.3 ± 0.3
Dexlo	X	4	1590 ± 200	0.53 ± 0.07
Dexlo	X	20	1560 ± 200	0.13 ± 0.02
Dexlo	Y	4	835 ± 100	0.09 ± 0.01
Dexlo	Y	20	—	—

[a] Adsorbent Y had a higher degree of cross-linking than adsorbent X as described under Materials and Methods.

[b] The values of P_{adm} were determined by taking the reciprocals of the intercepts at the ordinate of the lines described in Eq. [1].

[c] The values of K_a were calculated by multiplying the reciprocals of the slopes with B/P_{adm}.

lase and cross-linked starch as a reversible reaction, (b) we cannot discriminate between the binding sites of the adsorbent, (c) the α-amylase in solution is the only reagent that can react with those binding sites, and (d) there are no inhibitors that can react with α-amylase. In that case a straight line should be found when the reciprocal of the adsorbed enzyme activity is plotted against the reciprocal of the enzyme activity in the supernatant (5),

$$1/P_{ad} = (B/K_a P_{adm}) \times (1/P) + 1/P_{adm}. \quad [1]$$

However, inhibitors were present in the adsorbate, as shown by repeated adsorption from an α-amylase solution by refreshing the adsorbent. This resulted in a decreasing degree of adsorption with Maxamyl and Dexlo α-amylase. After six replacements of the adsorbent, however, the α-amylase activity in the supernatant was less than 1%, showing that practically all α-amylase can adsorb to the adsorbent. A_{sp} of the adsorbed α-amylase remained the same after each replacement. Therefore it is not likely that the decrease of adsorption was caused by the presence of several α-amylases with different adsorption characteristics in each enzyme mixture. The decrease in the degree of adsorption after each replacement was probably due to the accumulation of inhibiting contaminants. The degree of degradation of the adsorbent is 1.8% for 50 U/ml Maxamyl under adsorption conditions (4). The oligosaccharides, produced by the inevitable enzymatic degradation of starch, are, maybe with other inhibitors in the adsorbate, responsible for an aberration of an ideal equilibrium reaction.

However, by varying the initial enzyme concentrations in a specific range (Maxamyl, P_0 = 25–100 U/ml; Dexlo, P_0 = 100–400 U/ml) with equal amounts of adsorbent, straight lines were found if $1/P_{ad}$ was plotted against $1/P$. The margins of error of the extrapolated intercepts at the ordinate ($1/P_{adm}$) were relatively large. These effects continued in the calculated K_a values. Although the physical significance that can be attributed to K_a and P_{adm} remains obscure due to the inhibitors present in the adsorbate, there are significant differences between the values of K_a and P_{adm} in Table II.

The capacity of the adsorbent, which can be calculated from P_{adm}, did not change significantly by an increase in temperature (71 ± 11 mg/g for Maxamyl α-amylase and 185 ± 23 mg/g for Dexlo α-amylase with adsorbent X). Since both enzymes have about the same molecular weights one can conclude that for Dexlo α-amylase there are at least twice as many binding sites on cross-linked starch as for Maxamyl α-amylase.

The K_a values decreased with increasing temperature. Equilibrium experiments with a more stable cross-linked starch (Y) with less affinity for α-amylase (4) resulted in about equal K_a values and decreasing P_{adm} values for Maxamyl α-amylase. At 4°C with Dexlo α-amylase, the capacity of adsorbent Y decreased also compared with adsorbent X. In this case the calculated affinity constant K_a decreased about fivefold. At 20°C Dexlo α-amylase hardly adsorbs to adsorbent Y and no relation between P_{ad} and P was found.

Kinetic experiments of α-amylase with its normal substrate at increasing temperature, revealed values for K_m and V_{max}. Like the adsorption constant (K_a) these values also contain the reaction velocities involved:

$$k_1 = k_2/K_m + k_3/K_m \qquad [2]$$

$$V_{max} \sim k_3 \times E_T. \qquad [3]$$

In the Michaelis–Menten equation (Eq. [2]) K_m is not simply the dissociation constant for the enzyme substrate complex because it contains the k_3 term. The true dissociation constant (K_d) is k_2/k_1 which is the reciprocal of K_a. Since degradation products by enzymatic attack were measured we can consider the adsorption reaction of α-amylase to cross-linked starch similar to an enzyme substrate reaction. Now V_{max} (Eq. [3]) is very small compared with the normal enzyme–substrate system and the value of k_3/K_m can be neglected if the values of k_1 and k_2 are in the same order of magnitude with respect to the enzyme–substrate system. In that case K_m is the dissociation constant and the reciprocal of K_a. If only k_3 is affected by the modification of the starch polymers, and not k_1 and k_2, one could use the value of K_a from the adsorption experiments as an estimation for k_1/k_2 in the kinetics of the enzyme substrate system. With that approach it should be possible to calculate k_1 and k_2 separately in the kinetic experiments. However, to compare K_m (mg/ml) with K_a (liter/mol) an estimation of the molecular weight of the substrate should be made. It is not correct to use the molecular weight value with regard to the average length of the polymer since more than one enzyme molecule can attack the polymer chain at the same time. The value of k_2, the velocity constant of the backward reaction, was calculated for both enzymes at 4 and 20°C varying the length of the hypothetical substrate from 40 to 50 monomers (Table III).

This approach can only give a rough estimate of the k_2 values. However, the order of magnitude will be correct and the ratio of these values at different temperatures may still reveal useful information about the kinetics one may expect for the adsorption and desorption step. At 20°C the backward velocity constant increased about 30-fold for Dexlo α-amylase and 9-fold for Maxamyl α-amylase compared with the values at 4°C. The value of k_1, important in adsorption kinetics, increased at 20°C about 7-fold for Dexlo and 4-fold for Maxamyl α-amylase.

Thus, the value of k_2 increased more than the value of k_1 since the adsorption constant decreased at higher temperatures. This effect, and the fact that ionic strength

TABLE III

Calculated k_2 Values for Dexlo and Maxamyl α-Amylase at Two Different Temperatures

Enzymes	*T*	
	4°C	*20°C*
Dexlo α-amylase	1.8×10^{-4} (1.4×10^{-4})[a]	6.1×10^{-3} (3.5×10^{-3})
Maxamyl α-amylase	3.5×10^{-5} (2.7×10^{-5})	3.2×10^{-4} (2.4×10^{-4})

[a] A hypothetical substrate length of 50 monomer residues was taken to convert the values of K_m (mg/ml) to mol/liter. The values between parentheses were calculated with a substrate length of 40 monomers.

and pH do not have a significant influence on the degree of adsorption, justifies the conclusion that van der Waals interactions are the main interactions between bacterial α-amylases and the (modified) substrate (4, 15).

Improving the adsorption kinetics. Equilibrium experiments revealed that the degree of adsorption that can be achieved at equilibrium increases with decreasing temperature. Especially for Maxamyl but also for Dexlo α-amylase the initial reaction velocity is higher at 20°C than at 4°C (Fig. 3). However, the calculated k_1 values showed a larger increase of k_1 for Dexlo compared with Maxamyl α-amylase. Also the order of magnitude of the calculated k_1 values did not correspond with the experimental values. At 4°C equilibrium is not reached until 48 h, whereas at 20°C the degree of adsorption reached its maximal value after 16 h. Stepwise adsorption with decreasing

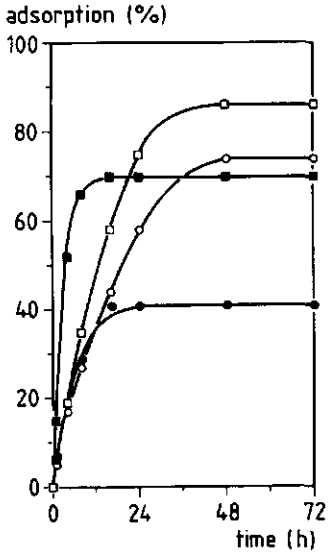

FIG. 3. Velocity of the adsorption of bacterial α-amylases by cross-linked starch in 0.1 M sodium acetate buffer (pH 6) at two temperatures. The original enzyme activity was 100 U/ml (○, Dexlo, 4°C; ●, Dexlo, 20°C; □, Maxamyl, 4°C; ■, Maxamyl, 20°C).

temperature to 4°C could be introduced to improve adsorption kinetics with an ultimate large degree of adsorption.

Adsorption kinetics were largely improved when the adsorbent was reused in a second adsorption–desorption cycle. The enzymatically modified adsorbent seemed to become more accessible for α-amylase. Adsorption experiments at low enzyme concentrations (below 25 U/ml) conducted with both enzymes at 4°C revealed that equilibrium was now reached within 1 h. The capacity of the adsorbent and the affinity constant toward α-amylase did not alter. Also the degree of degradation of this reused cross-linked starch was not changed significantly with respect to the original adsorbent.

In a subsequent paper (W. Somers *et al.*, unpublished results) it will be shown how adsorption kinetics were further improved.

Desorption Characteristics

Former investigations (4) showed that desorption of α-amylase could be realized by a raise in temperature. The equilibrium experiments, discussed above, showed that an increase in temperature resulted in an increase of the desorption constant. A disadvantage of the raise in temperature is the increased biodegradation rate of the adsorbent. Biodegradation should be avoided by minimizing the activity of the amylolytic system to achieve efficient desorption. A decrease of activity could be achieved by the use of 0.1 M succinate buffer, pH 6 (4) or simply by an increase in pH (e.g., 50 mM sodium carbonate, pH 10). However, preliminary experiments showed that with the addition of glycerol to the desorption buffer, biodegradation was sufficiently minimized (4). Since glycerol had a favorable effect on the stability of the enzymes and the amount of enzyme desorbed, we decided to use only the addition of 20% glycerol to the desorption buffer to realize a simple adsorption–desorption process.

To investigate desorption velocity, a loaded adsorbent was desorbed at different temperatures according to Materials and Methods (Fig. 4). For Dexlo α-amylase a degree of desorption of 50% could be achieved after 30 min at 40°C. At higher temperatures the enzyme activity in the supernatant decreased due to inactivation. Maxamyl α-amylase is reasonably stable at higher temperatures. The degree of desorption, however, was only 56% after 30 min at 70°C.

Biodegradation was avoided and the enzymes were effectively stabilized against irreversible thermal inactivation, by adding an amount of glycerol to the desorption buffer. An additional advantage was the higher degree of desorption that could be accomplished with 20% glycerol (v/v) in solution (Fig. 4). With glycerol the optimal desorption temperature for Dexlo α-amylase is about 60°C. There was thermal inactivation at 70°C. For Maxamyl α-amylase, no loss of activity was found at 70°C. For both enzymes, desorption was almost complete within 10 min.

DISCUSSION

The action patterns of two commercially available bacterial α-amylases which were used in our affinity adsorption experiments were determined. These were in good agreement with patterns known in the literature. It can be concluded that Dexlo α-amylase originates from a strain of *B. subtilis* or *B. amyloliquefaciens,* and Maxamyl α-amylase from a strain of *B. licheniformis.*

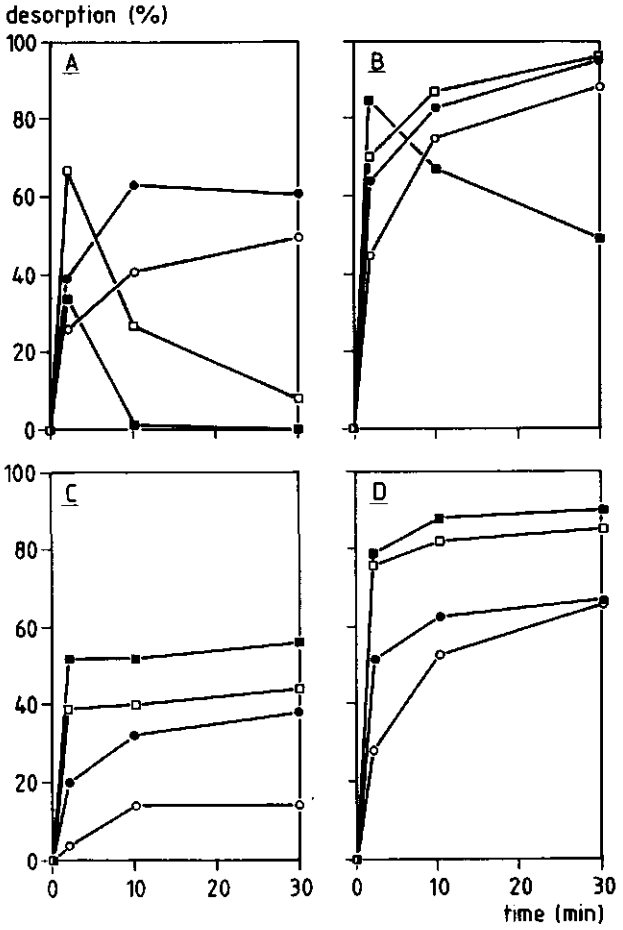

FIG. 4. Velocity of the desorption of bacterial α-amylases in the absence or the presence of 20% glycerol (Dexlo (A, B); Maxamyl (C, D)) from cross-linked starch in 0.1 M sodium acetate buffer at different temperatures (○, 40°C; ●, 50°C; □, 60°C; ■, 70°C).

Maxamyl and Dexlo α-amylase were purified on cross-linked starch with column chromatography. SDS–polyacrylamide gel electrophoresis and FPLC chromatography showed that the enzymes were free from contaminants present in the crude enzyme preparations.

Equilibrium studies were carried out with the cross-linked matrices to investigate adsorption capacities and adsorption constants as a function of temperature and type of adsorbent. Other parameters such as ionic strength and pH do not influence the degree of adsorption significantly (4). The adsorption of enzyme from a crude α-amylase preparation with a cross-linked starch adsorbent is not a simple reversible reaction. This is due to (a) partial degradation of the adsorbent, (b) the accumulation of oligosaccharides in the adsorbate thereby, and (c) possible inhibitors in the adsorbate. Nevertheless, because of the limited degradation of the adsorbent, Langmuir-type plots were still found if the initial enzyme concentration was varied within in a range of 25–100 U/ml for Maxamyl and 100–400 U/ml for Dexlo α-amylase. From the

reciprocal of these plots, values for P_{adm} and K_a could be calculated. The capacity of the adsorbent appeared to be related to the type of adsorbent and to the specific enzyme used. In the case of Maxamyl α-amylase the capacity did not depend on the temperature. The capacity of suitably cross-linked starch is about 71 mg/g adsorbent for Maxamyl and 185 mg/g adsorbent for Dexlo α-amylase (4°C). The affinity constants of both enzymes decrease with increasing temperature and are, at least for Maxamyl α-amylase, independent of the type of cross-linked starch.

Thus, for Dexlo α-amylase there are at least twice as many binding sites on cross-linked starch than for Maxamyl α-amylase. Since for both enzymes the molecular weights and the number of subsites are about the same, this difference in capacity is difficult to explain. An explanation could come from the inhibitory properties of oligomers toward these enzymes, but these data are not available for *B. licheniformis*. It is also possible that more of the binding sites are accessible to *B. subtilis* enzyme because of a difference in shape between the two enzymes molecules.

Comparison of our adsorption equilibrium data with the kinetic data of the α-amylases with their normal substrate showed that for Maxamyl α-amylase the calculated k_2 value at 20°C is 9 times higher than k_2 at 4°C. This difference is even larger with Dexlo α-amylase (about 30 times). This is in agreement with the fact that for Dexlo α-amylase a larger initial desorption velocity was measured at lower temperatures compared to Maxamyl α-amylase. The calculated values of k_1 were not in agreement with the initial adsorption velocity rates measured. The adsorption velocity appeared to be largely dependent on the accessibility of the cross-linked starch. The velocity of the adsorption reaction did increase when the adsorbent was used in a second adsorption–desorption cycle. Probably there is a barrier in the cross-linked starch particle that makes diffusion of the α-amylase to the binding sites more difficult. This barrier can be partially removed by enzyme action on the matrix. As will be shown in a subsequent paper (W. Somers *et al.*, unpublished results), the accessibility of the adsorbent can be also highly improved in a different way.

Desorption of bacterial α-amylases from cross-linked starch can be achieved easily by an increase in temperature. At higher temperatures, however, increased biodegradation of the adsorbent occurs. The amylolytic activity of the enzymes released in the desorption buffer has therefore to be minimized. One approach is to use 0.1 M sodium succinate (pH 6) as a desorption buffer. In that case Maxamyl α-amylase activity is reduced to 15% and Dexlo α-amylase to 40% compared to the activity in a 0.1 M sodium acetate buffer (pH 6) (unpublished results). Another approach is to raise the pH which affects strongly the activity of α-amylase but hardly the association between the enzyme and cross-linked starch. In a sodium carbonate buffer, pH 10, Maxamyl α-amylase activity was reduced to about 25% of the value measured in a sodium acetate buffer (pH 6.0). The decrease of Dexlo α-amylase activity at pH 10 was even higher (unpublished results). The pH effect on α-amylase adsorption at higher enzyme concentrations will be the subject of further investigations.

However, the method finally chosen to avoid biodegradation was the addition of 20% glycerol (v/v) to the desorption buffer. Additional advantages of this method were an increased thermostability of the enzymes and an increased degree of desorption. This enhanced desorption is probably due to competitive α-amylase affinity for glycerol. Polyols have been found to be competitive inhibitors for α-amylase. With fungal α-amylase from *Aspergillus oryzae* a relation has been observed between the

affinity for the active site of α-amylase and the thermal protective effect at high concentrations of different polyols (including glycerol) (16).

Without thermal inactivation Dexlo and Maxamyl α-amylase were almost completely desorbed within 10 min at 60 and 70°C, respectively. The optimal desorption temperature for Maxamyl α-amylase may be even higher than 70°C.

It may be concluded that some important conditions to make cross-linked starch an economically feasible adsorbent for bacterial α-amylases are fulfilled. The enzymes are adsorbed in sufficiently high amounts and it is possible to improve adsorption velocity considerably. Association between bacterial α-amylase and cross-linked starch is due to van der Waals interaction and is affected strongly by temperature changes. Complete desorption can be achieved at high temperatures if glycerol is added to the desorption buffer.

APPENDIX: NOMENCLATURE

A_{sp}	Specific activity (U/mg)
K_m	Michaelis–Menten constant (mg/ml)
k_1	Velocity constant of enzyme–substrate (enzyme–adsorbent) formation from the enzyme and its substrate (adsorbent) (liters/(mol · min))
k_2	Velocity constant of enzyme-substrate (enzyme–adsorbent) breakdown leading to free enzyme and the original substrate (adsorbent) (min^{-1})
k_3	Velocity constant of enzyme–substrate breakdown leading to free enzyme and hydrolyzed products (min^{-1})
E_T	Total enzyme concentration in kinetic experiments (Maxamyl α-amylase, 0.019 mmol/liter; Dexlo 0.009, mmol/liter)
P_0	Enzyme activity in solution before the addition of the adsorbent (U/ml)
P	Enzyme activity of supernatant after the addition of the adsorbent (U/ml)
P_{ad}	Enzyme activity bound on the adsorbent (U/ml)
P_{adm}	Maximal adsorbed enzyme activity, the maximal value that is obtained for P_{ad} as P_0 is increased until P_{ad} does not change (U/ml)
K_a	Adsorption equilibrium constant (liters/mol).
B	Enzyme activity per mmole protein (Maxamyl, 1.32×10^7 U/mmol; Dexlo, 2.75×10^7 U/mmol).

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Chapter 5

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Chapter 5

Crosslinked xylan as an affinity adsorbent for endo-xylanases

Summary

In order to facilitate the purification of xylanases from *Aspergillus niger*, an affinity adsorbent has been developed from oat spelts xylan. A suitable adsorbent was only obtained by crosslinking oat spelts xylan with epichlorohydrin in water but not in ethanol or ethanol water mixtures. After some initial degradation of the adsorbent (approximately 4%), no significant biodegradation was measured with a reused adsorbent. Up to 60% of the xylanase activity from an *Aspergillus niger* enzyme mixture (50 mU/ml) was adsorbed at pH 4 (50 mM sodium acetate buffer). The degree of adsorption to crosslinked xylan of four fractions of this preparation, previously separated by DEAE-Biogel A chromatography, varied between 40 and 90%.

Adsorption was strongly dependent on pH and ionic strength and desorption was easily accomplished by an increase in ionic strength. In addition to xylanases, polygalacturonases were also adsorbed to the matrix. No significant adsorption of ß-D-xylosidase, α-L-arabinofuranosidase, ß-D-galactosidase, ß-(1,4)-galactanase, ß-(1-3/6)-D-galactanase or cellulase activities was found.

Introduction

Xylanases play an important role in the decomposition of plant waste material by microorganisms. Significant improvement in the economics of bioconversion of biomass to fuels and chemicals can be achieved if the hemicellulosic component of biomass, which includes xylan, is utilized (Woodward, 1984). This application, and also the liquefaction of fruits and vegetables, requires the joint activity of xylanase and cellulolytic and pectinolytic enzyme systems (Biely, 1985). Cellulolytic enzymes must be absent if xylanases are used to remove xylan from wood pulp in the paper manufacturing process. In this way the xylan of the pulp can be converted to a mixture of sugars with economic value whereas the alkaline extraction of pulp results in xylan containing effluent streams (Paice & Jurasek, 1984).

Due to its complex structure, complete breakdown of naturally occurring branched acetylated xylan requires the action of several hydrolases. Important are endo-ß-(1,4)-xylanase (EC 3.2.1.8) that degrades the xylan backbone and ß-xylosidase (EC 3.2.1.37) that converts xylo-oligosaccharides to D-xylose. The xylan backbone, however, is not completely accessible to xylanases. The enzymes α-D-glucuronidase and α-L-arabinofuranosidase are required to remove sugar residues from side chains of the xylan backbone. An acetylesterase should be present to remove acetyl groups from the xylose residues in the polymer backbone.

We are interested in developing methods whereby these enzymes can be easily recovered from complex extracellular enzyme mixtures. Ideally, affinity chromatography could provide a single step procedure to isolate a particular enzyme. Xylanase from *Irpex lacteus* was purified by pseudo-affinity chromatography (hydrophobic interaction, dye ligand (Hoebler & Brillouet, 1984)). However, an affinity method would be more specific if xylan could be used as an adsorbent, since it can be recognized by the desired enzymes. Modification of original substrates by crosslinking with epichlorohydrin resulted in suitable adsorbents with pectate (endo-polygalacturonase (Rexová-Benková & Tibensky, 1972), endo-pectate lyase (Visser *et al.*, 1979)), starch (α-amylase (Weber *et al.*, 1976 and Rozie *et al.*, 1991)) and mannan (1,2-α-mannosidase (Tanimoto *et al.*, 1986)). In this paper the development of a suitable adsorbent for xylanase, by crosslinking of xylan with epichlorohydrin, is described.

Materials and Methods

The xylans used to prepare crosslinked adsorbents were from oat spelts (batch no. X-0376 and 107-F-0802 from Sigma, St.Louis, Missouri, USA) and from larch (batch no. 90581 from Koch & Light, Haverhill, UK). Oat spelts xylan (batch no. X-0376) was used to determine xylanase activity.

Sodium pectate obtained from Sigma (batch no. P-1879) was used to determine polygalacturonase activity. The content of polygalacturonic acid was 85-90%.

CMC-cellulose (Akucell type AF 0305, Akzo, Arnhem, the Netherlands) was used to determine cellulase activity.

Coffee arabino-ß-(1,3/6)-D-galactan, isolated from green coffee beans (*Coffea arabica*) according to the method of Wolfrom & Patin (1965), was used to determine ß-(1,3/6)-D-galactanase. Potato arabino-ß-(1,4)-D-galactan, isolated from destarched potato fibre according to the method of Labavitch *et al.* (1976) was used to determine ß-(1,4)-D-galactanase activity.

p-Nitrophenyl derivatives of α-L-arabinofuranose (Sigma), ß-D-galactopyranose and ß-D-xylopyranose (Koch & Light) were used to determine glycosidase activities.

The pectinolytic enzyme mixture KPB 00I 3XL from *Aspergillus niger*, obtained from Novo Ferment AG (Basel, Switzerland), was used in adsorption and degradation experiments. From preliminary experiments it was known that this enzyme mixture contained a considerable amount of xylanase activity (166 U/ml).

Determination of sugar composition

The uronide content was estimated using the method of Ahmed & Labavitch (1977) using glucuronic acid as a standard. The neutral sugar composition was determined by gas chromatography after hydrolysis according to Seaman *et al.* (1963) and derivatization to alditol acetates (Jones & Albersheim, 1972).

Elemental analysis

Elemental analysis of the original oat spelts xylan and a crosslinked oat spelts xylan was conducted with a Carlo Erba CHN Elemental Analyzer (type 1106, Milan, Italy).

Determination of xylanase and other polysaccharide splitting activities

Xylanase activity was determined with a modified ferricyanide test (Rozie *et al.*, 1988). The reaction mixture contained 100 µl of 0.5% (w/v) oat spelts xylan in 50 mM sodium acetate buffer (pH 5.0), 350 µl of this buffer without substrate and 50 µl of an enzyme solution (0-50 mU/ml). The mixture was incubated for 60 min at 30 °C. The reaction was stopped by the addition of 500 µl of 2% sodium carbonate after which the mixture was cooled in an ice bath. Next, 2 ml of a freshly prepared mixture (1:1) of a cyanide solution (0.25% KCN, 1% Na_2CO_3) and a ferricyanide solution (0.08% $K_3Fe(CN)_6$, 1% Na_2CO_3) was added. After standing for 20 min at room temperature, the reaction mixture (3 ml) was immersed in a boiling-water bath for 10 min and then immediately cooled in an ice bath. Discoloration was measured spectrophotometrically at 420 nm after 1 h. The absorbance changes were interpreted in terms of an increase in the concentration of reducing sugars by means of a calibration curve for D-xylose. One unit (U) was defined as the amount of enzyme which released 1 µmol of reducing groups per min.

Determinations of other endo-enzyme activities such as polygalacturonase, cellulase, ß-(1,3/6)-D-galactanase and ß-(1,4)-D-galactanase were conducted in a similar way. Instead of a 0.5% (w/v) xylan solution, 0.5% (w/v) solutions of the desired substrates, described above, were used. For all these enzymes the calibration curve for D-xylose was used. Calibration curves for various sugars differ by 5-15% from that obtained for xylose.

Determination of ß-D-xylosidase, ß-D-galactosidase and α-L-arabinofuranosidase activities

For the determination of ß-D-xylosidase activity 50 µl enzyme sample (0-20 mU/ml) was mixed with 350 µl 50 mM sodium acetate buffer (pH 5). Next, 100 µl 0.1 % *p*-nitrophenyl-ß-D-xylopyranoside was added. The reaction mixture was incubated for 1 h at 30 °C. The reaction was stopped by adding 500 µl 500 mM glycine buffer (pH 9; 2 mM EDTA). The absorbance changes were measured spectrophotometrically at 400 nm. One unit (U) was defined as the amount of enzyme which released 1 µmol of *p*-nitrophenol per min .

Determinations of ß-D-galactosidase and α-L-arabinofuranosidase activities were conducted in a similar way. Solutions of 0.1 % *p*-nitrophenyl-ß-D-galactopyranoside and *p*-nitrophenyl-α-L-arabinofuranoside were used, respectively.

Protein determination

Protein determinations were performed with the Lowry method and measured spectrophotometrically at 690 nm (Lowry *et al.*, 1951). To spare sample, small amounts of proteins (150 µl; 0-50 µg/ml) were determined with this test in a micro titerplate scanner EAR 400 (SLT GmbH, Salzburg, Switzerland). Lowry reagent (50 µl) was prepared by mixing an alkaline solution (4% sodium carbonate in 1.0 M sodium hydroxide) with a copper solution (2% copper (II) sulfate . 5 H_2O) and a tartrate solution (4% potassium sodium tartrate) in the ratio of 46:2:2 .

Determination of the matrix biodegradation

The biodegradability of the adsorbent was determined by measuring the total amount of saccharides in the supernatant after incubation of the adsorbent with an enzyme mixture. According to the method of Dubois *et al.* (1956) 900 µl phenol (2.5 % w/v) was added to a 100 µl sample. The solution was mixed and then 2.5 ml sulphuric acid (96%) was added. The reaction mixture was mixed again thoroughly. The solution was cooled for 1 h at ambient temperature. The colour was measured spectrophotometrically at 490 nm. The absorbance changes were interpreted by means of a standard graph for the original xylan (0-1 mg/ml) and corrected for the enzyme blank. The percentages of degradation were corrected for the increased weight of the crosslinked xylan with respect to the original polysaccharide.

Synthesis of crosslinked xylans in ethanolic solvents

In 10 ml test tubes amounts of ethanol (96%) or ethanol/water (1:1) were added to portions of 200 mg oat spelts xylan (Sigma, X-0376) in such a way that the final total reaction volume was always 2 ml. Amounts of epichlorohydrin (12-60 µl) and 5 M NaOH in water (30-152 µl) were added to the reaction mixtures. A molar NaOH/epichlorohydrin ratio of 1.0 was maintained. The test tubes were shaken for 24 h at 30 °C. The suspensions were neutralized with 5 ml 7% acetic acid and centrifuged after which the supernatant was decanted. The residue was washed successively with 5 ml water (twice), ethanol (twice) and acetone (twice).

Synthesis of crosslinked xylan in water

Oat spelts xylan and larch sawdust xylan were crosslinked in water in different reaction volumes, keeping the amounts of reagents constant. Water (0.706 - 3.484 ml)

and 5M NaOH (1.52 ml) were added to portions of 500 mg xylan in 20 ml test tubes. The tubes were agitated with a Vortex mixer to homogenize the brownish sludges. Epichlorohydrin (0.59 ml) was added while the reaction mixtures were agitated. The reaction was allowed to proceed for 22 h at 40 °C. To mix the epichlorohydrin thoroughly with the other reagents the tubes were agitated for 15 sec in time spans of 5 min at the start of the reaction (1 h). The gels obtained were cut with a spatula, neutralized with 10 ml 7% acetic acid and centrifuged after which the supernatants were decanted. The gel particles were washed five times with 10 ml water. Dehydration occurred by freeze-drying.

Synthesis of crosslinked xylan suitable for adsorption experiments

8.88 g oat spelts xylan was suspended in 26.54 ml 5M NaOH and 12.52 ml water in a 100 ml flask. The suspension was stirred with a magnetic stirrer. The magnetic follower had a diameter of 10 mm and a length of 45 mm. In some cases the viscous suspension had to be homogenized with a spatula. The mixture acquired a dark brown colour on the addition of NaOH. At 40 °C 10.55 ml epichlorohydrin was added. Magnetic stirring became easier because of the increase of the volume of the reaction mixture. After 15-30 min the mixture gelatinized and stirring was no longer possible. The reaction continued for 24 h at 40 °C. Next the temperature was increased up to 70 °C (for about 16 h). The gel was crushed with a spatula and homogenized in a Waring blender 801E (model 32BL80, Dynamics Corp., New Hartford , USA). The fine particles were transferred to a 1 litre flask. About 1 litre 7% acetic acid was added. The mixture was stirred thoroughly after which the particles settled down. After decantation of the supernatant and the finest particles, the residue was washed 3 times with 1 litre of water. After that a brown-yellow powder was isolated by lyophilization (yield 12.4 g). This product was used in adsorption studies.

Enzymatic degradation of adsorbents

10 mg of crosslinked xylan was incubated for 20 h at 25 °C with 1 ml KPB xylanase (50 mU/ml) in 50 mM sodium acetate (pH 4) in an Eppendorf centrifuge tube. The adsorbent was centrifuged and the degree of degradation was measured from the xylan dissolved in the supernatant. The degree of adsorption was calculated from the xylanase activity in the supernatant. After washing of the adsorbent (1 ml 500 mM NaCl (twice), 1 ml water (4 times)) and subsequent lyophilization, the remaining

adsorbent was exposed again to the enzyme in a similar incubation.

Composition of enzymatically degraded (crosslinked) xylan

Oat spelts xylan and its crosslinked derivatives were suspended (10 mg/ml) in 0.05% KPB xylanase in 50 mM sodium acetate buffer pH 4.0 . The enzyme mixture had been previously dialyzed to remove interfering saccharides. The xylans were incubated for 48 h at 30 °C. The breakdown products were determined by HPLC (SP 8000, Spectraphysics, San José, USA) equipped with a CH-Pb column (300 x 7.8 mm, Merck, Darmstadt, FRG) and a guard column (50 x 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, Richmond, USA). The analytical column was operated at 85 °C, the guard column at ambient temperature. Elution was done with water at a flow rate of 0.4 ml/min. Sugars were detected with a Shodex refractive index detector.

Effect of degraded crosslinked xylan on xylanase activity

An aliquot of 100 µl KPB was added to a suspension of 0.5 g crosslinked xylan (Table 3, 150 mg/ml) in 10 ml 50 mM sodium acetate buffer (pH 5.5). The testtube was rotated at room temperature for 24 h after which the material was centrifuged. The supernatant with the degraded polysaccharides was heated for 5 min at 100 °C. The effect of this material on xylanase activity was measured in the normal xylanase activity assay.

Influence of pH and ionic strength on adsorption

Portions of 10 mg crosslinked xylan were incubated with 1 ml of a KPB solution (50 mU/ml) in 50 mM sodium acetate buffer of different pH values (pH 4-6) in Eppendorf centrifuge tubes. Experiments to study the influence of the ionic strength were performed at pH 4. The NaCl concentration in the buffer was varied in the range 0-200 mM. The solutions were mixed for 20 h at 25 °C in a test tube rotator. Next, the adsorbent was centrifuged and the degree of adsorption of the xylanase activity was determined taking the difference between the total enzyme activity and the activity in the supernatant.

Separation of different xylanase activities by DEAE chromatography

50 ml KPB commercial enzyme solution was desalted with a Biogel-P10 column

(800 x 45 mm) in 20 mM sodium acetate buffer (pH 5). 10 ml of the eluate (in total 110 ml) was separated on a DEAE-Biogel-A column (150 x 40 mm) in 50 mM sodium acetate buffer (pH 5) using a stepwise increase in NaCl concentration.

Influence of pH on xylanase activity of KPB and DEAE purified fractions

Xylanase activity of the crude KPB xylanase and of the fractions purified by DEAE column chromatography was measured as described above. The buffers used in the assay were 100 mM sodium acetate (pH 3.0; pH 4.0; pH 5.0; pH 6.0), 50 mM sodium phosphate (pH 7.0; pH 8.0; pH 9.0) and 50 mM sodium carbonate (pH 10.0). The pH of the assay solutions was measured again after addition of the enzyme sample (50 mM sodium acetate, pH 5.0). Percentages of activity were plotted taking the activity at pH 5.0 as 100 % .

Adsorption of KPB and of DEAE-Biogel-A separated fractions on crosslinked xylan

Portions of 10 mg crosslinked xylan were incubated with 1 ml of an adsorbate (50 mU/ml xylanase activity) in 50 mM sodium acetate buffer (pH 4 and pH 5) in an Eppendorf centrifuge tube. The solutions were mixed for 20 h at 25 °C in a test tube rotator. Next, the adsorbent was centrifuged and the degree of adsorption of enzyme was calculated from the enzyme activity in the supernatant.

Results

Composition of xylan

In our attempts to synthesize a suitable adsorbent for xylanases from the natural substrate of the enzyme, we used two xylans. One originated from larch sawdust, the other from oat spelts. The saccharide composition of these complex carbohydrates was determined after hydrolysis and derivatization to alditol acetates (Table 1). Relative to larch xylan, oat spelts xylan contained less xylose and mannose and more glucose and arabinose. Determination of the amount of reducing groups (Rozie *et al.*, 1988) revealed for both xylans an average size of 150 residues per molecule. However, the linear xylose backbone is known to carry a large number of side groups. According to Reilly (1981) larchwood xylan is a linear chain of xylopyranose residues, in which every fifth or sixth residue is substituted at C-2 with a 4-O-methyl-D-glucuronic acid

Table 1

Sugar composition of the xylans used in adsorbent synthesis.

Sugar	Larch sawdust xylan mol %	Oat spelts xylan mol %
Arabinose	7.8	10.4
Xylose	76.7	72.1
Mannose	3.1	0.5
Galactose	1.3	1.5
Glucose	5.2	9.3
Uronic Acid[a)]	5.9	6.2

[a)] Uronic acid is expressed as glucuronic acid.

unit and in which a small number of xylopyranose residues is substituted at C-3 with arabinofuranose. In view of the lower xylose content it may be presumed that oat spelts xylan is more branched than larch wood xylan.

Crosslinking in an ethanolic solvent

In preliminary experiments oat spelts xylan was crosslinked in an ethanolic solvent. The epichlorohydrin concentration and the ethanol/water ratio were varied. The solvent composition itself had no effect on the properties of the product with respect to its degradability by xylanase. However, an increase of the epichlorohydrin concentration in the crosslinking reaction resulted in products with a higher degree of crosslinking as is reflected by the reduced biodegradability when the crosslinked xylans were incubated with a commercial xylanase preparation (Table 2). The product prepared with the lowest epichlorohydrin concentration (76 μmol/ml) dissolved completely with a xylanase enzyme mixture. Although the crosslinked xylans prepared in ethanolic solvents cover a broad range of more or less degradable matrices, in none of the cases was enzyme adsorption measured. Thus it seems that this lack of adsorption cannot be attributed to an unfavourable degree of crosslinking. It may be due to the low accessibility for xylanases of the relatively hard and compact adsorbents prepared in ethanolic solvents.

Table 2

Degree of enzymatic degradation of crosslinked xylans prepared in an ethanolic solvent relative to unmodified xylan.

Epichlorohydrin concentration (μmol/ml)	Solvent	
	Ethanol degradation (%)	Ethanol/water (1:1) degradation (%)
0	100	100
76	93	91
152	40	39
228	8.0	8.1
304	5.6	6.8
380	2.0	4.1

Crosslinking of xylan in water

Next, crosslinking in a larger effective reaction volume was carried out to achieve high accessibility of the adsorbent. In an ethanolic solvent the reaction is heterogeneous. Intermolecular and intramolecular crosslinking of the polysaccharide chains with glyceryl bridges is very likely to occcur as the chains are reasonably close to each other. Crosslinking of polysaccharides solubilized in water, e.g. dextran (Flodin, 1962), starch (Kuniak & Marchessault, 1972) and arabic gum (Fujita *et al.*, 1975) resulted in gelation of the total reaction mixture. In those cases the polysaccharide is homogeneously distributed over the total reaction volume. This volume is an important parameter in determining the degree of crosslinking and finally the accessibility of the modified polysaccharide to enzymes.

Crosslinking in water was carried out both with oat spelts and larch sawdust xylan. More epichlorohydrin (2 moles per mole xylose monomer) was used since preliminary experiments showed that with regard to the crosslinking in ethanolic solvent higher amounts of epichlorohydrin were necessary to prepare a product which was less than 10% degradable by xylanase activity. The total reaction volume was varied (2.8 - 5.6 ml), keeping the amount of epichlorohydrin constant. The resulting gels were weaker with increasing reaction volume. The gels were crushed, neutralized and washed. Subsequent dehydration with ethanol resulted in hard stone-like particles. A powder was obtained if dehydration of the water washed gel particles was carried out by

freeze drying.

Portions of 10 mg of the adsorbents prepared in this way were incubated with 1 ml of a KPB solution (50 mU/ml) in 50 mM sodium acetate buffer (pH 5) in an Eppendorf centrifuge tube and mixed for 20 h at 25 °C in a test tube rotator. The modified xylans were centrifuged and the degree of degradation of the adsorbents was calculated from the xylan material in the supernatant. In some cases limited degradation and appreciable adsorption of xylanase activity was found (Table 3).

Larch sawdust xylan cannot be crosslinked as effectively as oat spelts xylan (Table 3), probably because of the difference in composition and in average chain length.

Table 3

Properties of crosslinked xylans from oat spelts and larch sawdust prepared in water solvent.

Xylan concentration[a] (mg/ml)	Yield[b] (%)	Elemental composition[c] C (%)	H (%)	Degree of degradation (%)	Degree of adsorption (%)
Oat spelts xylan					
100	173			43	
125	165	44.7	6.6	41	
150	173			34	
175	146	45.0	6.8	11	12 (31)[d]
200	136			3	28 (55)
225	138	46.5	7.0	4	29 (59)
Larch sawdust xylan					
100	22				
125	132			98	
150	121			85	
175	144			56	
200	137			48	
225	111	42.9	6.5	39	

[a] Mg/ml xylan in the crosslinking reaction mixture before the addition of epichlorohydrin.

[b] Yield after freeze drying as percentage of the original xylan.

[c] Elemental analysis was carried out for some modified xylans. In all cases 0% nitrogen was detected. The carbon and hydrogen content of the orginal xylans were: C 41.5%, H 6.4% for oat spelts xylan ; C 40.2%, H 5.9% for larch wood xylan. The margin of error in these measurements is 0.3%.

[d] Values are given for adsorption at pH 5 and at pH 4 (in parenthesis), both in 50 mM sodium acetate.

The low yield at the largest reaction volume is due to the high solubility of the product in the washing fluids. All crosslinked larch xylans were degraded by xylanase and no adsorption of enzyme was found. The yields with regard to the original polysaccharide of the crosslinked xylans that did not dissolve in water were relatively high.

The concentrations of xylan in Table 3 could not be increased further because of mixing problems with the extremely viscous solutions. Also the amount of epichlorohydrin added to the reaction mixtures could hardly be increased. Larger amounts of epichlorohydrin did not mix properly with the other reagents, thus forming a two phase system. The values in Table 3 show that the degradation of an adsorbent by xylanase was minimized if oat spelts xylan was crosslinked in water in a reaction volume as small as possible. In that case adsorption of xylanase to the matrix could be detected. The synthesis on a larger scale of such a crosslinked xylan suitable for adsorption studies was carried out under vigorous stirring in a 100 ml flask. The disruption of the gel and the washing procedures were the same as those used to prepare Sephadex (Flodin, 1962).

The elemental composition of this product was shown to be equal to that of the product prepared on a smaller scale (xylan concentration 225 mg/ml, Table 3). The carbon content was increased compared to the original oat spelts xylan. From the yield of the crosslinking reaction (140 %) one can calculate that about one glyceryl moiety is present per sugar monomer. In that case the high carbon content of the modified xylan is in agreement with a fully crosslinked xylan, in which every hydroxyl group is connected to another hydroxyl group by a glyceryl diether bridge. However, these calculations are based on a hypothetical xylan that consists of D-xylose residues, with two available reactive hydroxyl groups, only. One can expect an increase in carbon content from 40.0 % to 46.6 % if this hypothetical xylan is fully crosslinked by epichlorohydrin. In fact, the increase in carbon content, due to the modification of oat spelts xylan, was not that high (from 41.5 % to 46.5 %). This means that a part of the glyceryl moieties is present as glyceryl monoether residues. With increasing reaction volume there is an obvious trend of increasing yield and decreasing carbon content. This is caused by a shift from glyceryl diether bridges to glyceryl monoether side chains in crosslinked xylan. From the yield (173 %) of a modified oat spelts xylan, crosslinked at a lower xylan concentration (100 mg/ml), it can be calculated that c. 1.5 glyceryl moieties are present per sugar monomer. In that case only a small part

of the glyceryl moieties is present as a connecting bridge between polysaccharide chains. Such an adsorbent is apparently easily degraded by enzymatic action, although most of the hydroxyl groups in the crosslinked xylan are substituted.

Polysaccharides, present in the supernatant of the degraded crosslinked xylan (150 mg/ml), had no inhibitory effect on the xylanase activity of KPB-enzyme. This shows that the dissolved polysaccharide fragments are hardly recognized by the xylanase enzymes, which may be primarily due to the high substitution grade of those polysaccharides.

Composition of enzymatically degraded crosslinked xylan

Oat spelts xylan and its crosslinked derivatives were degraded as described in Materials and Methods. The supernatants with the solubilized polysaccharides were injected on HPLC. Xylose was the main component (80%) in the supernatant of the degraded unmodified oat spelts xylan. The remainder consisted of 7% arabinose and two oligosaccharides (3% and 8%). The supernatants of the degraded crosslinked oat spelts xylans showed on HPLC a large amount of oligosaccharides which overshadows the relatively small xylose peak (3-10%). Arabinose peaks were detected but could not be quantified in this material. From these results it is noticeable that, although it was measured only in the degraded material, there is an amount of unsubstituted xylan present in the crosslinked oat spelts xylan.

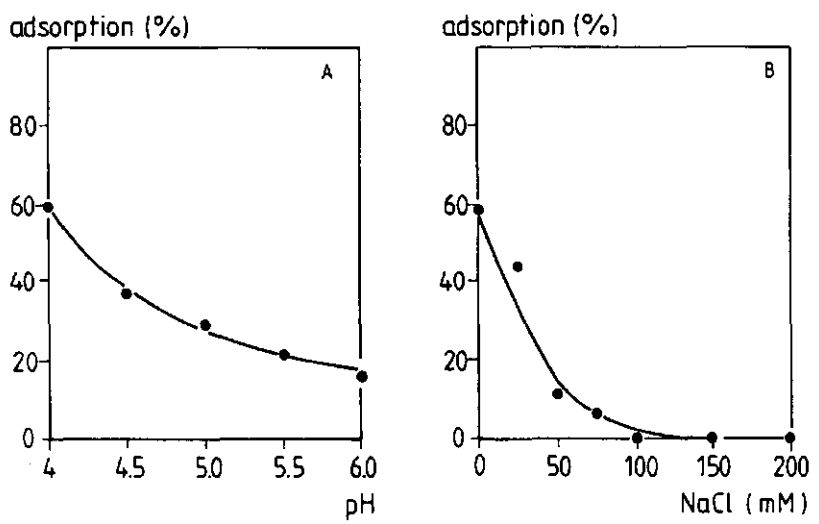

Fig. 1 Adsorption of xylanases with A) pH and B) ionic strength (pH 4) in 50 mM sodium acetate buffers. The original xylanase activity was 50 mU/ml. The amount of adsorbent was 10 mg/ml.

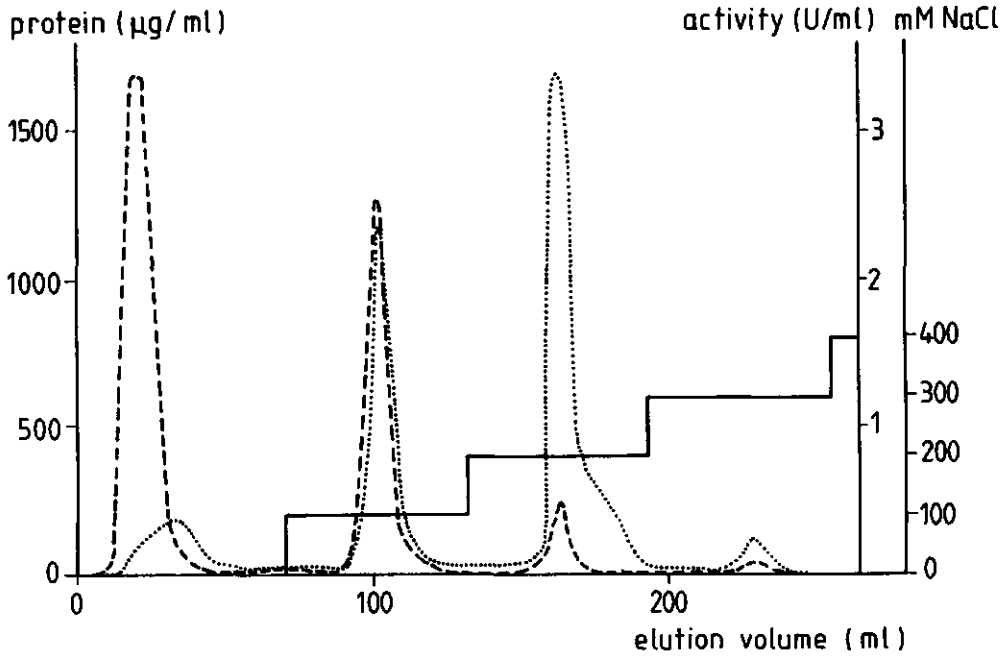

Fig. 2 Separation of different xylanase activities by DEAE chromatography. (..... protein (µg/ml), ---- xylanase activity (mU/ml), —— NaCl concentration(mM))

Repeated degradation

About 4% of the adsorbent, prepared on a larger scale as described above, was degradable by xylanase activity. The degree of adsorption of KPB xylanase was 60%. The adsorbent was reused in a similar degradation experiment after a washing procedure and subsequent lyophilization. No significant degradation was measured with the reused adsorbent. The degree of adsorption did not change significantly (63%).

The influence of pH and ionic strength on adsorption of A. niger xylanase

The influence of pH was studied with sodium acetate buffers at a low ionic strength (Fig. 1a). Adsorption was maximal at pH 4, the lowest pH studied, although the optimal pH for enzyme activity of crude KPB xylanase was 5.0 . Adsorption was also studied in 50 mM sodium citrate buffers of pH 3.0, 3.5 and 4.0. However, with 50 mM sodium citrate buffers no significant adsorption was found. Fig. 1b shows that this is due to the higher ionic strength of the citrate buffer solutions (54 mM at pH 4) compared to the sodium acetate buffers (7.4 mM at pH 4). The addition of 50 mM

NaCl to the acetate buffer reduces the adsorption to approximately 10%.

Separation of different xylanase activities by anion-exchange chromatography

Since there are different xylanases present in fungal extracellular enzyme preparations (Dekker & Richards, 1976), these activities were separated in a conventional way to study the adsorption properties of the individual xylanases to the crosslinked xylan affinity adsorbent. Therefore xylanases present in KPB were treated by anion-exchange chromatography using DEAE-Biogel-A (Fig. 2). Four fractions with xylanase activities were pooled. The fraction which was not bound by the column material contained about 80% of the xylanase activity and some other endo-polysaccharide splitting activities (e.g. polygalacturonase). This xylanase had no activity at pH 3.4 contrary to the xylanases that were eluted from the column with the stepwise salt gradient (Fig. 3). Enzyme activities found in the different fractions are shown in Table 4. The xylanolytic activities of fractions I to IV did not add up to that of the crude KPB preparation: the yield was only 66%. This effect can be explained partly by synergistic phenomena which are known to exist when these enzymes are allowed to work simultaneously (Dekker & Richards, 1976).

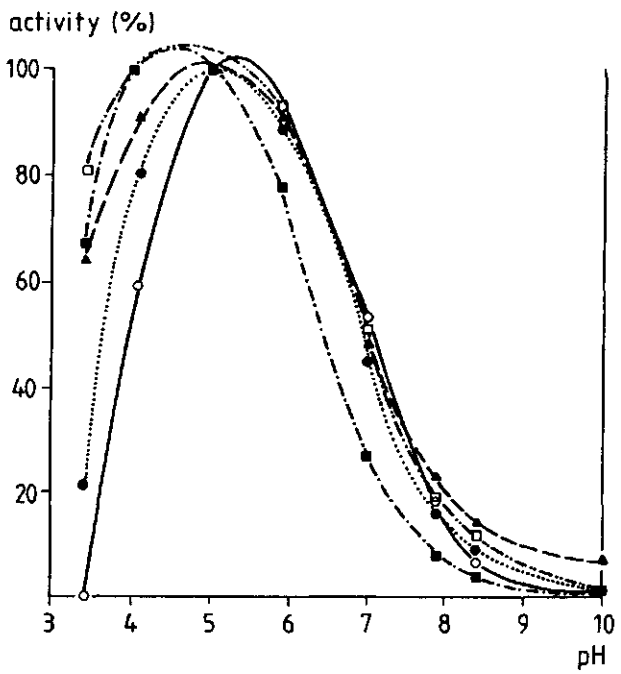

Fig. 3 pH dependency of xylanase related to their activity at pH 5. (● crude KPB, ○ fraction I, ■ fraction II, □ fraction III, ▲ fraction IV)

Adsorption of crude KPB and DEAE-separated enzyme fractions to crosslinked xylan

The crude commercial xylanase preparation and the xylanase fractions separated by DEAE chromatography were incubated with crosslinked xylan as described in Materials and Methods. The xylanase fraction I that was not retained by the DEAE-Biogel-A column, did not adsorb to crosslinked xylan at pH 5 but it did bind to the adsorbent at pH 4. Xylanase activity from the fractions II and III was adsorbed at pH 5 but binding did improve at pH 4. With fraction IV there was a slight decrease in the degree of adsorption at a lower pH.

In these adsorption experiments it was found that ß-D-xylosidase, α-L-arabinofuranosidase and ß-D-galactosidase activity did not adsorb significantly to the crosslinked xylan adsorbent. There was also no adsorption for endo activities such as cellulase, ß-(1,3/6)-D-galactanase and ß-(1,4)-D-galactanase. Apart from xylanase, only some polygalacturonase enzyme activity did adsorb to the modified xylan. Polygalacturonase behaved similar to xylanase as it was also bound to a larger extent at pH 4 than at pH 5. The fractions I to IV showed decreasing degrees of polygalacturonase adsorption (Table 5).

Table 4

Protein content and enzyme activities in fractions pooled after DEAE-Biogel-A chromatography of KPB enzyme.

	Fraction			
	I	II	III	IV
Protein (mg)	11.0	39.0	73.5	24.9
Xylanase (U)	420	71	11	1.8
Polygalacturonase (U)	3200	1900	730	173
Cellulase (U)	2.7	3.7	33	11
ß-(1,3/6)-D-Galactanase (U)	6.4	31	280	69
ß-(1,4)-D-Galactanase (U)	0.97	57	28	63
ß-D-Xylosidase (U)	n.d.*	7.1	8.5	0.27
α-L-Arabinofuranosidase (U)	n.d.	0.96	490	160
ß-D-Galactosidase (U)	n.d.	190	39	2.6

* n.d. = not detected.

Table 5

Degrees of adsorption of crude and DEAE-purified xylanases on crosslinked xylan.

	Degree of adsorption		
	Xylanase pH 4 (%)	Xylanase pH 5 (%)	Polygalacturonase pH 4 (%)
KPB	60	29	84
Fraction I	91	0	90
Fraction II	58	15	72
Fraction III	72	40	54
Fraction IV	41	49	45

Discussion

Modification of polysaccharides, such as pectate (Rexová-Benková & Tibensky, 1972) and starch (Weber *et al.*, 1976), by crosslinking these polymers with epichlorohydrin in water as solvent resulted in products which could still adsorb enzymes involved in the breakdown of such polysaccharides. These adsorbents, however, were still partly degraded by these enzymes. Better reproducibility and a higher effective degree of crosslinking was obtained for pectate (Visser *et al.*, 1979) and starch (Rozie *et al.*, 1991) when the crosslinking procedure was conducted in an ethanolic solvent. When the polysaccharide was more effectively crosslinked, there was a decrease of adsorbent biodegradation together with a decrease in the degree of adsorption of the substrate splitting enzymes. For crosslinked starch some biodegradation was inevitable if suitable adsorption properties were to be achieved (Rozie *et al.*, 1991).

A similar strategy was thought to be necessary in order to prepare crosslinked xylan for the adsorption of xylanases. However, it appeared that oat spelts xylan cannot be crosslinked in an ethanolic solvent to an adsorbent that binds xylanases. This is probably due to a limited accessibility of the crosslinked polymer chains. Accesibility

cannot be improved by grinding the crosslinked polymers to a powder (0.5 mm). However, by dissolving xylan in water and by crosslinking it in a homogeneous reaction, useful adsorbents were obtained. High concentrations of xylan were required in the reaction mixture to get a sufficiently high degree of crosslinking, in which biodegradation was limited (≤ 4%). The product yield of the crosslinking reaction and elemental analysis of xylan and modified xylan showed that a large number of the available hydroxyl groups in xylan were crosslinked. HPLC analysis of enzymatically degraded crosslinked xylan showed however that there are still xylose residues present that are not substituted. Adsorption of xylanase (60%) was measured with a crosslinked xylan. The same degree of adsorption but no further biodegradation was found if the adsorbent was used in a second adsorption experiment.

No suitable adsorbent could be prepared with larch sawdust xylan, although this material had a higher xylose content than the suitable oat spelts xylan. The latter material is expected to have high degrees of substitution as was concluded from the large product yields. With decreasing reaction volume, larch sawdust xylan shows a similar decrease in product yield as oat spelts xylan probably due to a shift from mono- to di-substituted glyceryl. However, the large product yields, and with that the apparently necessary degree of substitution, are not reached with larch sawdust xylan. The low glyceryl content compared to oat spelts xylan is confirmed by elemental analysis.

Since there are different xylanases present in the commercial enzyme preparation, these activities were separated by anion exchange chromatography to study the adsorption of the individual enzymes to the matrix separately. From the four xylanase fractions that were obtained, one xylanase (I) adsorbed for more than 90% to the adsorbent prepared at pH 4. With this isolated xylanase no adsorption on crosslinked xylan was found at pH 5. It differs from the others as it has no enzyme activity on the original substrate at pH 3.4 . None of the other xylanases obtained by DEAE chromatography had these two features, although the adsorption of the xylanases in fraction II and III was improved at pH 4 compared to that at pH 5. The xylanase in fraction IV adsorbed better at pH 5.

About 80% of the xylanase activity, eluted from the DEAE-Biogel-A column, is present in fraction I. A large amount (90%) of this xylanase and reasonable amounts (41-72 %) of the xylanases in the other fractions adsorb to the matrix. Of the original enzyme preparation only 60% of the activity adsorbs to the adsorbent. This difference

can be due to synergism between the enzymes that remain in the adsorbate or to inhibitory components in the crude enzyme preparation.

Crosslinking of heteropolymers as xylan could result in an adsorbent with affinity for many enzymes of the complex enzyme mixture that has activity on the original xylan. It appeared from the enzymes involved in xylan degradation that only endo-xylanases were bound to the synthesized adsorbent.

Adsorption experiments were also conducted for cellulase and polygalacturonase since these activities mostly occur in commercial fungal enzyme mixtures in which xylanase activity is found. Polygalacturonase activity was adsorbed by the matrix, cellulase was not. The adsorption of polygalacturonase, an enzyme that does not have activity on the natural xylan, is probably due to the uronic acid content of xylan, which acts thereby as an ion exchange matrix.

Polygalacturonase and xylanase adsorption decreased when the ionic strength is decreased. Also, xylanase adsorption was less at 4 °C compared to the values shown at 25 °C (results not shown). These results show that xylanase adsorption by crosslinked xylan is due primarily to electrostatic interactions between the enzyme and the adsorbent. The question may be raised whether this association is due to ion exchange properties of the adsorbent or to biomolecular recognition of the xylan backbone. Investigations in that matter will be discussed in another paper as well as capacity and possible applications of the adsorbent.

Acknowledgements

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Chapter 6

Chapter 6

Crosslinked xylan as a tool for the purification of endo-xylanases from complex enzyme mixtures

Summary

Crosslinked xylan, prepared by the reaction of oat spelts xylan with epichlorohydrin, was used as an affinity adsorbent for endo-xylanases. Four commercial fungal enzyme preparations were tested for their binding behaviour on crosslinked xylan. Adsorption was tested in 50 mM sodium acetate, pH 4.0. Desorption of the adsorbed enzymes was easily accomplished by an increase in ionic strength. Besides endo-xylanases also endo-polygalacturonase enzymes were adsorbed by crosslinked xylan, probably due to the D-glucuronic acid moieties in xylan. The xylanase activity in Pectinol A1 (Röhm GmbH, Darmstadt, Germany) was efficiently purified with crosslinked xylan. The specific endo-xylanase activity increased from 5.5 U/mg up to 160 U/mg. Two proteins were found with SDS-PAGE in purified Pectinol (29 and 51 kD) whereas a K_m of 1.1 mg/ml was measured. Equilibrium adsorption studies revealed a rather low capacity for the Pectinol endo-xylanase (1.5 mg xylanase/g adsorbent). The calculated K_a was 4 x 10^6 L/mole.

Some endo-xylanases were also adsorbed by cation exchange material. However, from crosslinked xylan chromatography and additional FPLC studies it appeared that the adsorption properties of crosslinked xylan were not only due to the cationic properties of this adsorbent.

Introduction

Lignocellulose is an abundant and renewable resource with cellulose, hemicellulose and lignin as the major constituents (Kennedy & Melo, 1990). Hemicellulosic material, including xylan, is an important carbon reservoir in nature. In the biodegradation process of this complex polysaccharide by micro-organisms a number of enzymes is involved, each with its own substrate specificity (Wong *et al.*, 1988). Technological significance is expected for xylan degrading enzymes, especially when they are free of cellulase (Biely, 1985; Tan *et al.*, 1987). As both enzyme systems are produced by most fungi, an inexpensive process should be available for the separation of the xylanase system from the cellulase system (Tan *et al.*, 1987).

Ideally, biospecific affinity adsorbents could provide such a simple separation procedure. An affinity adsorbent for xylanases that is nonselective with respect to cellulases is wood powder (Fujishima *et al.*, 1989). More selective xylanase adsorption is suggested with immobilized substrate oligomers (Call *et al.*, 1987). Affinity adsorption is found with an apple endo-1,4-ß-xylanase using an immobilized dye (Suzuki & Ashida, 1989).

Modification of the natural substrate of the xylanase system seemed a suitable way to prepare an affinity adsorbent for the xylanase system. Modification of xylan by crosslinking with epichlorohydrin yields a polymer that has been investigated for its flame retardancy properties (Simkovic *et al.*, 1990) and for selecting xylanase producing strains (Zemek *et al.*, 1981). In our study a crosslinked deacetylated xylan was used as an enzyme adsorbent. Xylan is partly acetylated in nature and the enzymatic digestibility with fungal endo-xylanases, like those of *Trichoderma reesei* is strongly affected by the degree of acetylation (Mitchell *et al.*, 1990). In most cases acetyl esterases are necessary in nature to make the xylan biopolymer accessible to endo-xylanases (Biely, 1985). Thus, only part of the enzymes that build up the xylanase system of a microorganism are expected to have interaction with the deacetylated xylan that we used as the original material. It was expected that chemical crosslinking of this material would lead to an adsorbent that was useful to isolate endo-xylanases from complex enzyme mixtures.

In a previous paper (Rozie *et al.*, 1992) we prepared a crosslinked xylan that adsorbed some endo-xylanase and endo-polygalacturonase activity from an *Aspergillus niger* enzyme mixture, whereas biodegradation of the modified polymer was limited

to about 4%. No biodegradation was measured when the crosslinked xylan was used repeatedly. As adsorption of enzyme activities on crosslinked xylan was due to electrostatic interactions, desorption was easily performed by a slight increase in ionic strength.

However, other endo-xylanases, polygalacturonases and also cellulases, galactanases and exo-enzymes such as ß-xylosidase and α-L-arabinofuranosidase did not adsorb to the affinity adsorbent. Crosslinked xylan is therefore not a universal adsorbent to isolate the complete xylanase system. The use of crosslinked xylan as an inexpensive adsorbent in on-off affinity chromatography to isolate particular endo-xylanase activities from fungal enzyme mixtures is described in this Chapter.

Materials and Methods

The xylan used to prepare the affinity adsorbent was obtained from Sigma (oat spelts, batch no. 107-F-0802, St.Louis, Missouri, USA). Sigma oat spelts xylan (batch no. X-0376) was used to determine xylanase activity.

Sodium pectate obtained from Sigma (batch no. P-1879) was used to determine polygalacturonase activity. The content of polygalacturonic acid was 85-90%.

Enzyme preparations used were KPB 00I 3XL and Pectinex 3XL AP18 (both from Novo Ferment AG, Basel, Switzerland) and Rohament CT and Pectinol A1 (both from Röhm GmbH, Darmstadt, FRG). The former two originate from *A.niger*, whereas the source of the Röhm preparations is unknown.

Determination of xylanase and polygalacturonase activities

Xylanase activity was determined with a modified ferricyanide test (Rozie *et al.*, 1992). One unit (U) was defined as the amount of enzyme which released 1 μmole of reducing end groups per min. A calibration curve of D-xylose was used.

Polygalacturonase activity was determined in a similar way (Rozie *et al.*, 1988). Instead of a 0.5% (w/v) xylan solution, a 0.5% (w/v) pectate solution was used as a substrate to measure enzyme activity. A calibration curve of D-galacturonic acid was used.

Synthesis of crosslinked xylan

The synthesis of a suitable crosslinked xylan has been described (Rozie *et al.*, 1992). Starting with 6.63 g oat spelts xylan a brown yellow powder of crosslinked xylan was obtained (yield 8.84 g).

DEAE chromatography of KPB xylanase.

As described previously (Rozie *et al.*, 1992) KPB xylanase was separated in 4 different fractions by DEAE chromatography. 50 ml KPB commercial enzyme solution was desalted with a Biogel-P10 column (800 x 45 mm) in 20 mM sodium acetate buffer (pH 5). Part of the eluate (10 ml from 110 ml) was chromatographed on a DEAE-Biogel-A column (150 x 40 mm) in 50 mM sodium acetate buffer (pH 5) using a stepwise increase in NaCl concentration which leads to the 4 fractions mentioned.

Chromatography on crosslinked xylan

Lyophilized crosslinked xylan (7.5 g) obtained a volume of 52 ml after hydration in 50 mM sodium acetate buffer (pH 4.0) and was then loaded in a glass column (15 x 350 mm). Pectinol A1 (50 mg in 0.5 ml of the same buffer) was chromatographed using a flow of 0.5 ml/min. After 100 min, the buffer applied was replaced by a 50 mM sodium acetate buffer (pH 4.0) containing 500 mM NaCl. This buffer was applied for 150 min to desorb all components that were bound to the crosslinked xylan. Fractions of the eluate (1 ml) were analysed for xylanase and polygalacturonase activities. In relevant fractions the protein content was determined. Concentrations higher than 500 mM M NaCl were not applied as 1 M NaCl did modify the structure of the column material. The shrinkage and clogging of the adsorbent resulting from high salt concentrations was irreversible.

The next enzyme sample could be purified in a similar way by washing the column first with 100 ml 50 mM sodium acetate buffer (pH 4.0). However, Pectinex and KPB from Novo Ferment AG were liquid preparations. To obtain sufficient adsorption, these enzyme mixtures were first diluted ten-fold with 50 mM sodium acetate (pH 4.0) and then dialysed against the same buffer. Aliquots of 1 ml of these dialysed samples were used for column chromatography.

FPLC analysis of crude preparations and purified xylanase fractions

The crude enzymates and the fractions isolated with crosslinked xylan were separated on a Mono-S cation exchanger using FPLC equipment (Pharmacia, Uppsala, Sweden). The chromatography procedure was carried out with a 200 μl loop using a 50 mM sodium acetate buffer (pH 4.0) with a gradient of 0-0.5 M sodium chloride and a flow of 1 ml/min . Xylanase activity was measured in eluate fractions of 0.5 ml.

Determination of protein content

Protein determinations were performed with the Lowry method and measured spectrophotometrically at 690 nm (Lowry *et al.*, 1951). To minimize the sample used, small amounts of proteins (150 μl; 0-50 μg/ml) were determined with this test in a micro- titerplate scanner (Rozie *et al.*, 1992).

SDS-PAGE electrophoresis and FPLC patterns

Electrophoresis of crude and fractionated enzyme preparations was carried out with the Phast-system™ apparatus (Pharmacia, Uppsala, Sweden). Marker proteins (low mol. wt. standards) were obtained from Bio-Rad (Richmond, USA). The gel was coloured with Coomassie brilliant blue (PS™ Development Technique file no. 200).

Preparation of degraded crosslinked xylan.

500 mg of crosslinked xylan was incubated for 20 h at 25 °C with 50 ml Pectinol A1 xylanase (1 mg/ml) in 50 mM sodium acetate (pH 4.0) in an Eppendorf centrifuge tube. The product was isolated after washing with 50 ml 500 mM NaCl (2x) and 50 ml water (4x)) and by lyophilization.

Reduction of crosslinked xylan and uronide determination

To a suspension of crosslinked xylan (0.21 g) in 10 ml water 1 mmole of solid CMC[1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-p-toluenesulfonate]was added which was obtained from Aldrich Chemie (Bruxelles, Belgium). The initial pH after stirring was pH 4.2 . As the reaction proceeded the pH increased to 4.45 . After 1 h 200 μl HCl was added whereafter the pH decreased to 4.25. The reaction was allowed to proceed for 2 h. 15 ml of 2M sodium borohydride was added slowly to the reaction mixture at room temperature whereafter the pH rose to 8.6. In contrast to a similar procedure followed by Taylor & Conrad (1972) the pH was not maintained

at pH 7 at this stage. After 1 h the solid material was washed by decantation with 1 L 50 mM sodium acetate (pH 4.0, 2x) and 1 L water (2x). After centrifugation and subsequent lyophilization of the modified crosslinked xylan, the yield was 190 mg. The uronide content of the original and the modified crosslinked xylan was estimated using the method of Ahmed & Labavitch (1977) using D-glucuronic acid as a standard. The uronide determination was not disturbed by the neutral sugar (D-xylose) in the polymer material.

Rate of xylanase adsorption.

Portions of 10 mg crosslinked xylan were incubated in Eppendorf centrifuge tubes in three series with 1 ml of a Pectinol A1 solution in 50 mM sodium acetate buffer (pH 4.0). In series a and b crosslinked xylan was used which was not enzymatically pretreated. In series c enzymatically pretreated crosslinked xylan was used as an adsorbent. The starting enzyme concentration was 125 μg/ml for series a, and 1 mg/ml for series b and c. The solutions were mixed at 25 °C in a test tube rotator for several time spans between 10 min and 1200 min. Next, the adsorbent was centrifuged and the degree of adsorption of the xylanase activity was determined by calculating the difference between the total enzyme activity and the activity in the supernatant.

Adsorption at equilibrium

Crude enzyme (Pectinol A1) and a Pectinol A1 xylanase preparation purified on a crosslinked xylan column were used. The initial enzyme concentration (0.5 - 4 U/ml) was varied in a series of five batches to determine the degree of adsorption under defined conditions. The concentration of crosslinked xylan was 10 mg/ml in 50 mM sodium acetate buffer (pH 4.0). The suspensions (7.5 ml) were agitated at 22 °C in a test tube rotator. The amount of enzyme bound at equilibrium was determined as the difference in enzyme activity of the initial enzyme solution and the activity in the sample taken from the supernatant after 18 h.

Adsorbent degradation.

The biodegradability of the adsorbent was determined by measuring the total amount of saccharides (Dubois *et al.*, 1956) in the supernatant after incubation of the adsorbent (10 mg/ml) with an enzyme mixture (Pectinol, 1 mg/ml) in 50 mM sodium acetate buffer (pH 4.0), as described previously (Rozie *et al.*, 1992).

Michaelis Menten constant of purified Pectinol xylanase.

Enzyme kinetics were investigated for Pectinol A1 xylanase that was purified on a crosslinked xylan column (xylanase activity 375 mU/ml; specific activity 150 U/mg).

Xylanase activities were measured at various concentrations of partially dissolved oat spelts xylan (0.07 - 2.0 mg/ml) in 4.5 ml of 50 mM sodium acetate buffer (pH 4.0) which contained 100 µl of the purified enzyme solution. The xylanase concentration was 8.3 mU/ml. The mixtures were incubated and stirred at 30 °C for 20 min. With 5 min intervals, samples were taken and analysed for reducing sugars. The Michaelis-Menten constant (K_m) and V_{max} of the endo-xylanase were calculated from a Lineweaver-Burke plot.

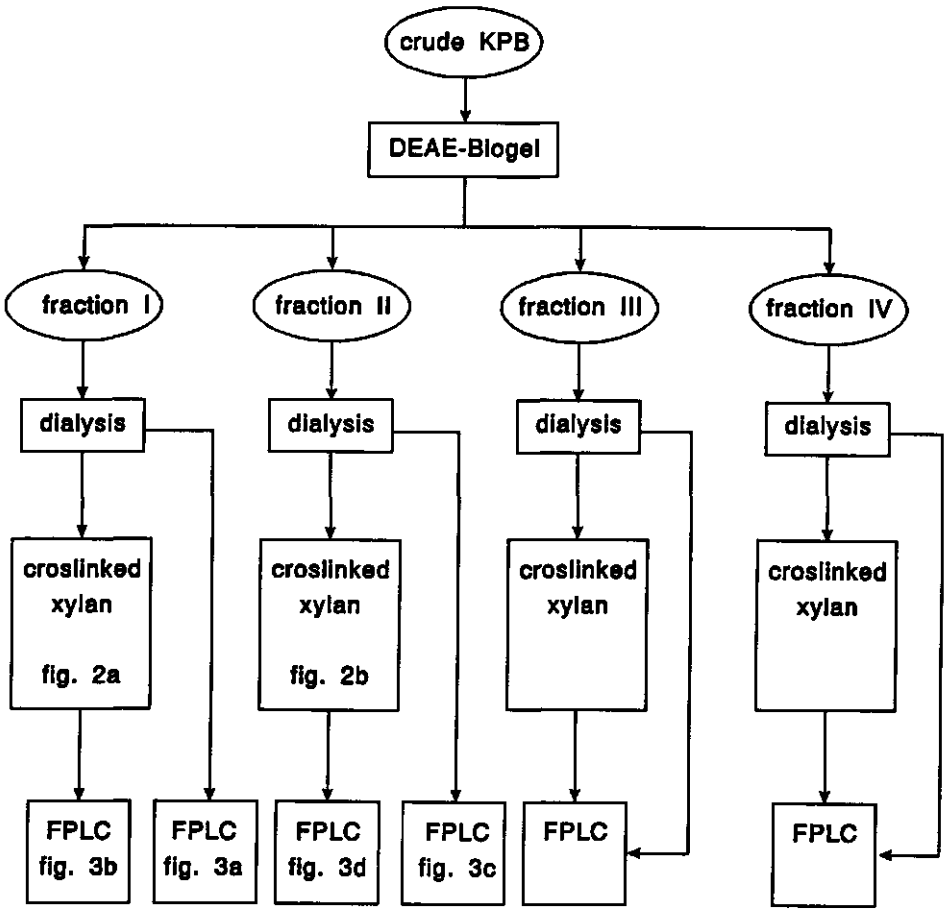

Fig. 1 Schematic presentation of the purification steps that are used to separate xylanase activities in crude KPB enzymate.

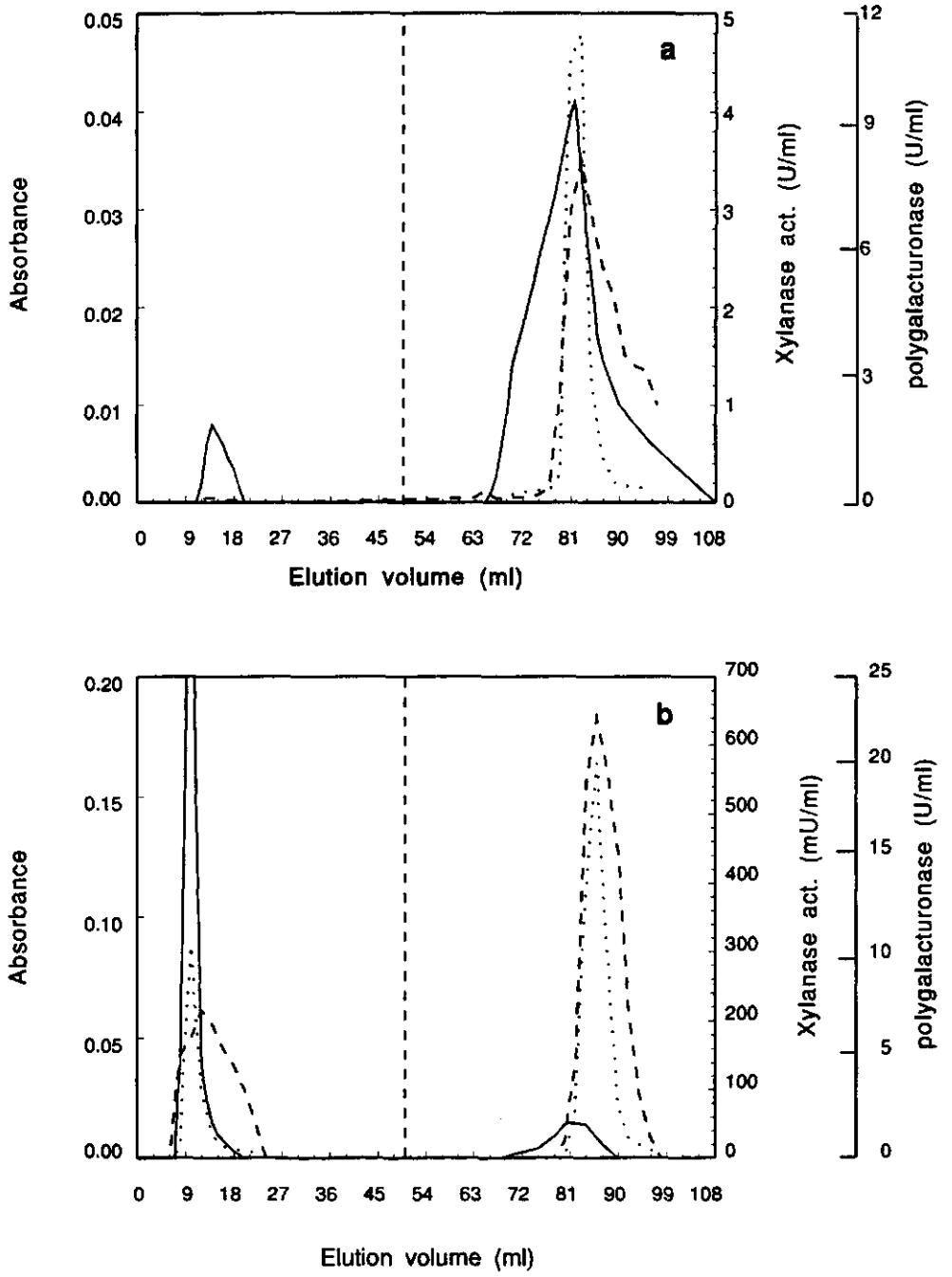

Fig. 2 Chromatography of DEAE-separated xylanase containing enzymates originating from KPB on a crosslinked xylan column: a) fraction I, b) fraction II (—— absorbance at 280 nm, - - - - xylanase activity, ... polygalacturonase activity; elution with 500 mM NaCl did start at 50 ml elution volume)

Results

Crosslinked xylan as an affinity adsorbent

Crosslinked xylan, prepared as described in the Materials and Methods section has a bed volume of approximately 6.9 ml/g. Crude enzyme preparations and fractions thereof were applied to a column with a 52 ml bed volume in a buffer of low pH and low ionic strength (50 mM sodium acetate, pH 4.0). Adsorbed components were desorbed with 500 mM NaCl in the same buffer. The column material was used repeatedly for more than 25 adsorption desorption experiments. Over that period a 10% reduction in bed volume was observed with this material.

Fractionation on crosslinked xylan and FPLC of different KPB enzyme fractions.

KPB enzymate is a fungal extracellular enzyme preparation containing several endo-xylanases. In a previous paper (Rozie *et al.*, 1992) it was reported that the KPB enzyme preparation could be separated into four xylanase fractions with DEAE chromatography. This conventional separation method seemed useful to study the adsorption properties of the individual xylanases to the crosslinked xylan affinity adsorbent. The procedure which was applied for the fractionation of the endo-xylanases in KPB is shown in Fig. 1. Fraction I was eluated directly from a DEAE-Biogel column. The other fractions were eluated with increasing salt concentrations. Fraction I contained less than 10% of the proteins in the eluate but more than 80% of xylanase activity and about 50% of polygalacturonase activity in the eluate. In Fig. 2a it is shown that the protein material of fraction I did bind almost completely to crosslinked xylan. Practically all xylanase and polygalacturonase activity was adsorbed on the column. Fraction II of the DEAE-separation contained about 25% of the protein material, about 15% of the xylanase activity and about 30% of polygalacturonase activity. This fraction contained also galactanases and a number of exo-enzymes such as ß-D-xylosidase and ß-D-galactosidase. The latter enzymes did not bind to crosslinked xylan (Rozie *et al.*, 1992). Compared to fraction I, a relatively large part of xylanase and polygalacturonase activitities did not bind to the column (Fig. 2b).

The DEAE-fractions III and IV contained respectively 50 and 16% of the total protein, 12 and 3% of the polygalacturonase activity and 2 and 0.5% of the xylanase

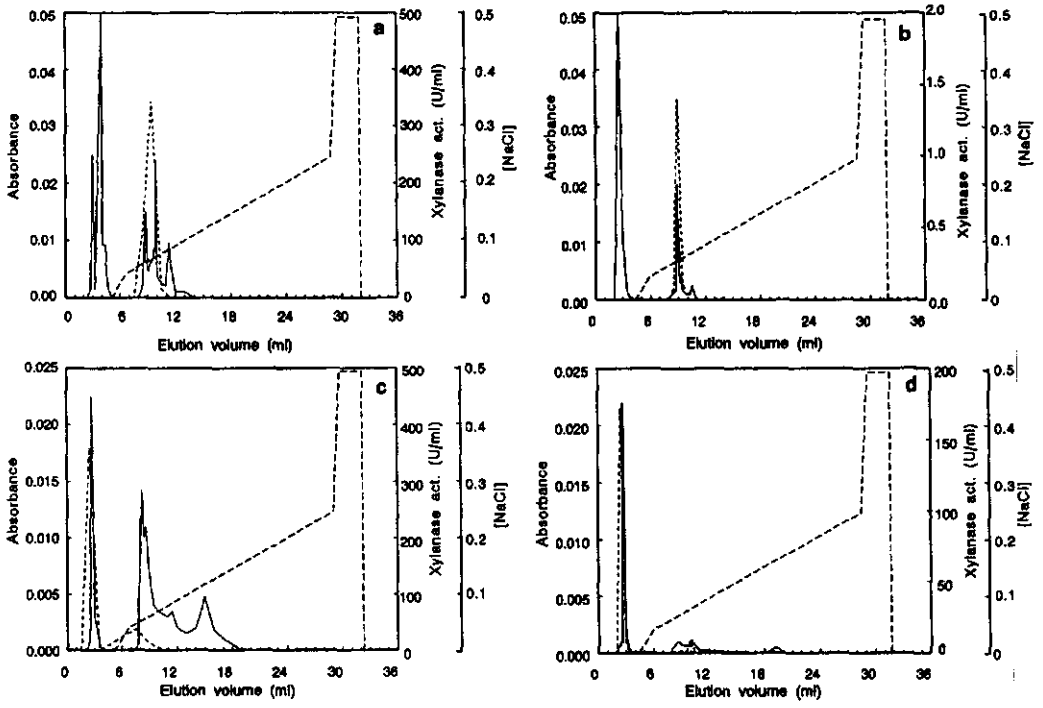

Fig. 3 Chromatography on a Mono-S column (FPLC) of DEAE-separated enzymates originating from KPB and the corresponding enzymates purified on a crosslinked xylan column: a) fraction I, b) purified fraction I, c) fraction II, d) purified fraction II (—— absorbance at 280 nm, --- xylanase activity, – – NaCl concentration).

activity. These two fractions contained most of the cellulases and α-L-arabinofuranosidases. The latter enzymes were hardly adsorbed by the crosslinked material (Rozie *et al.*, 1992). Again, part of both polygalacturonase (48% and 32% respectively) and xylanase activity (79% and 52% respectively) did bind to the crosslinked xylan adsorbent.

As shown in Fig. 1, FPLC was applied to the four fractions obtained by DEAE chromatography of KPB enzymate. Also four corresponding fractions, but now purified in advance on crosslinked xylan, were chromatographed with FPLC. For that purpose we used the fractions eluted under desorption conditions with the highest xylanase activity. All fractions were previously dialysed against 50 mM sodium acetate buffer (pH 4.0). The applied Mono-S column consists of cation exchange material as opposed

to the DEAE anion exchange column from which the fractions originated. The enzyme fractions that were eluted first from the DEAE-column (I,II) did contain the minor part of the protein from crude KPB. Most of these proteins in fractions I and II were adsorbed by the Mono-S column (Fig. 3a and 3c) contrary to the proteins of fractions III and IV. Protein material of fraction I was separated by cation exchange chromatography into different fractions. Xylanase activity was only detected in the eluate and divided over different protein peaks. Fraction I that was purified further with crosslinked xylan showed a FPLC pattern (Fig. 3b) in which xylanase activity was only detected in one fraction after 7 ml, apparently similar to the first peak in Fig. 3a. With SDS-PAGE a molecular mass of 36 kD was determined. Xylanase activity in fraction II was eluted largely in the void volume on FPLC (Fig. 3d) in contrast with its behaviour on crosslinked xylan (Fig. 2b). This phenomenon was more pronounced with fraction III. Only a small part of the xylanase activity was retained by the FPLC column. However, xylanase activity from fraction III, pre-purified on crosslinked xylan, was retained almost completely by the Mono-S material. These irregularities could be due to the fact that not all the xylanase containing fractions, desorbed from crosslinked xylan during one run, have to be composed equal to the one selected to chromatographe on FPLC. Moreover, the total composition of the enzyme mixture in which a particular enzyme is present seems to be of importance and the formation of enzyme complexes may for instance lead to trailing effects.

Practically all xylanase activity of fraction IV, whether pre-purified with crosslinked xylan or not, did elute in the void volume with FPLC.

Chromatography of different commercial enzyme preparations on crosslinked xylan.

Four different commercial enzyme preparations were tested at ambient temperature on crosslinked xylan. As Pectinex and KPB from Novo Ferment AG are liquid enzyme preparations of high ionic strength these were dialysed first against equilibration buffer (50 mM sodium acetate, pH 4.0). In Fig. 4a and 4b it is shown that some of the proteins and phenolic compounds were adsorbed by the adsorbent. Most of the xylanase activity was adsorbed in the case of Pectinex (79%) whereas with KPB this was slightly less (68%). Both preparations did contain a large amount of polygalacturonase activity as compared with Pectinol (KPB 3200 U/ml; Pectinex 4600 U/ml; Pectinol 420 U/g). With Pectinex and KPB, a relatively large portion of the polygalacturonase activity is adsorbed by crosslinked xylan (36% and 60%

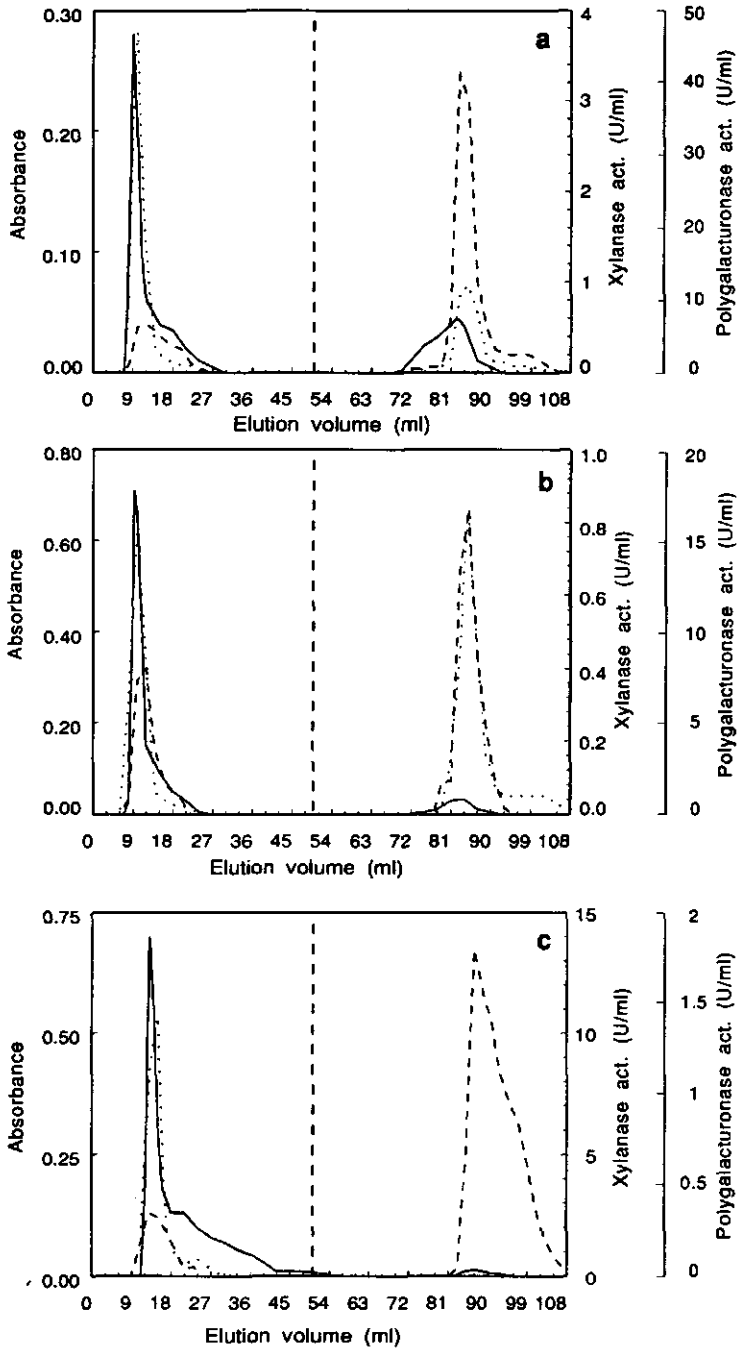

Fig. 4 Chromatography of several commercial enzymates on a crosslinked xylan column: a) Pectinex, b) KPB, c) Pectinol (—— absorbance at 280 nm, - - - xylanase activity, ····· polygalacturonase activity; elution with 500 mM NaCl did start at 50 ml elution volume)

respectively). With the desorption procedure applied the adsorbed polygalacturonase activity eluted in exactly the same fraction as the adsorbed xylanase enzymes.

Crude Pectinex xylanase activity is also divided by Mono-S chromatography in an adsorbing and a non-adsorbing part (Fig. 5a). Pectinex that was purified on crosslinked xylan showed no xylanase activity in the void volume on FPLC (Fig. 5d). The major protein bands of the two adsorbed xylanase fractions in Fig. 5a and 5d, as analysed by SDS-PAGE, corresponded with a molecular mass of 36 kD. Surprisingly, the retention time of the xylanase fraction of Pectinex purified by Mono-S slightly increased compared with the same fraction in crude Pectinex. A similar effect is obtained with the crude KPB enzyme preparation and xylanase of KPB that is first fractionated with crosslinked xylan. This effect may be due to interfering components in the crude enzyme preparations, although precautions were taken by previous dialysis of the crude enzymates. From Fig. 5d it is evident that Pectinex xylanase, purified on crosslinked xylan, is still contaminated with proteins without xylanase activity. No data are available about polygalacturonase activity in these fractions.

Crude Pectinex and crude KPB revealed FPLC chromatograms that are totally different with regard to xylanase activity and UV absorbance. However, the FPLC chromatography patterns of these two enzymates, pre-purified on crosslinked xylan, are similar to each other. As in Pectinex in KPB a major protein band corresponding with a molecular mass of 36 kD was found in the eluted enzyme fraction containing xylanase activity. This molecular mass corresponds with that found for the xylanase in the previously described DEAE-fraction I. However, the retention time of the xylanase with a molecular mass of 36 kD from fraction I (Fig. 3b) is not similar with the retention times found for the corresponding xylanases in Pectinex and KPB (Figs. 5d and 5e).

The two enzymates from Röhm GmbH (Pectinol A1 and Rohament CT) turned out to behave chromatographically identical. Also by other measurements, such as determination of protein content and xylanase and polygalacturonase activities, we were unable to discriminate between these two preparations. Chromatographed on crosslinked xylan most of the proteins and phenolic components present in these two enzyme preparations eluted in the void volume. Under desorption conditions material was eluted which had low UV absorption, a small protein content and high xylanase activity. The specific xylanase activity of this fraction as compared to the original enzyme solution was increased from 5.5 U/mg up to 160 U/mg . The Michaelis-

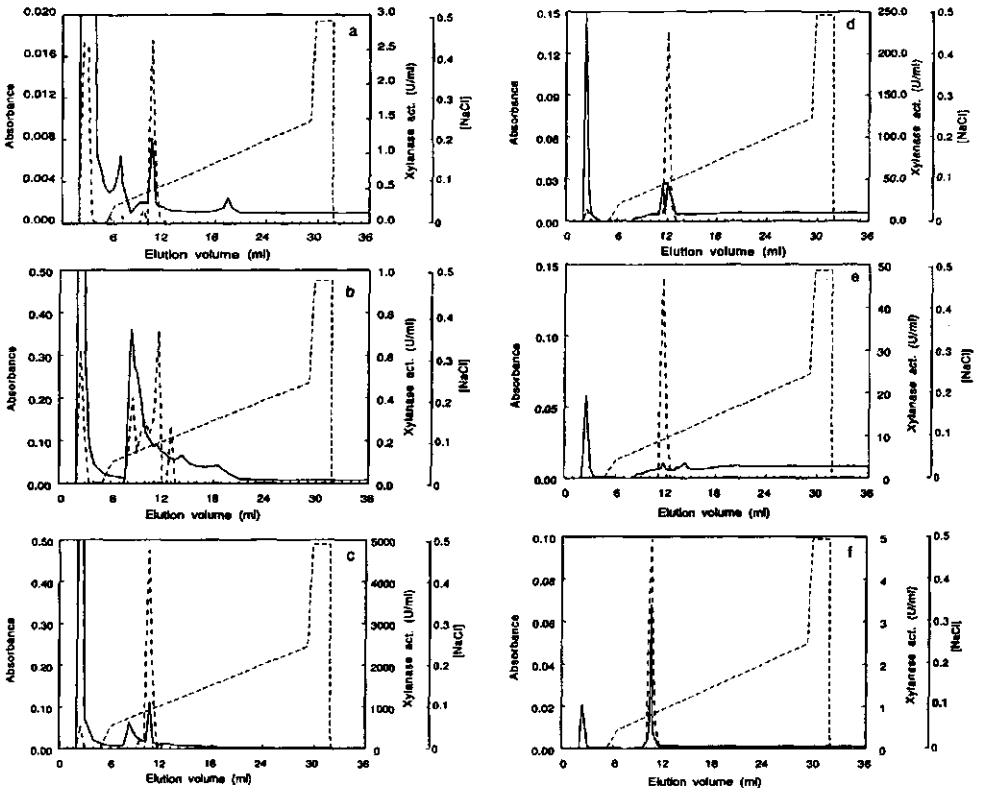

Fig. 5 Chromatography on a Mono-S column (FPLC) of crude enzymates and the corresponding enzymates purified on a crosslinked xylan column: a) crude Pectinex, b) crude KPB, c) crude Pectinol, d) purified Pectinex, e) purified KPB f) purified Pectinol (—— absorbance at 280 nm, --- xylanase activity, – – NaCl concentration).

Menten constant was 1.1 mg/ml (V_{max} = 307 U/mg). By SDS-PAGE analysis two proteins were found in this enzyme fraction with apparent Mw values of 29 and 51 kD. Pectinol A1 and Rohament CT contained less polygalacturonase activity in comparison to Pectinex and KPB and as shown in Fig. 4c this polygalacturonase is not adsorbed by the xylan adsorbent.

Only a small fraction of protein in Pectinol A1 was bound to the Mono-S cation exchange material. As shown in Fig. 5c, the peak that was eluted with a NaCl gradient contained practically all xylanase activity. Pectinol xylanase, purified with crosslinked xylan as described before, contained a small fraction of protein without xylanase activity that eluted directly from the Mono-S column. Another fraction corresponded

with the xylanase containing fraction from crude Pectinol. A fraction in crude Pectinol (after 8 ml) that did bind on Mono-S material was not present in Pectinol purified with crosslinked xylan.

Crude Rohament CT enzymate and a fraction hereof that was desorbed from crosslinked xylan showed the same FPLC patterns as obtained from crude and purified Pectinol A1.

Cation exchange properties of crosslinked xylan

There are some similarities between the adsorption properties of crosslinked xylan towards the complex enzyme mixtures and the adsorption properties of the FPLC cation exchange material. However, comparison of Figs. 2 and 3 reveals that in some cases Mono-S material does not adsorb xylanases that were adsorbed by crosslinked xylan. Also, some enzymes (eg. in Pectinol) that were adsorbed by the Mono-S material were not adsorbed by crosslinked xylan (Fig. 5).

Still, it is likely that some or all enzymes are adsorbed by crosslinked xylan not due to the molecular recognition aimed at, but simply because of the cationic properties of the xylan polymer. The latter might be due to the glucuronic acid content of xylan.

To study this possibility, it was attempted to eliminate the carboxyl groups from crosslinked xylan by reducing them with sodium borohydride according to the method of Taylor & Conrad (1972). Only a 40% reduction of the uronic acid content (from 6% to 3.5%) was accomplished.

The influence of this chemical modification on the adsorption properties of the material was tested in batch experiments with KPB enzymate similar to the experiments performed before (Rozie *et al.*, 1992). The reduced crosslinked xylan adsorbed the same amount of xylanase activity as the non-reduced material. The amount of polygalacturonase activity adsorbed decreased however with about 50%.

Rate of xylanase adsorption and adsorbent degradation.

Two concentrations of Pectinol were incubated with an amount of crosslinked xylan as described in Materials and Methods. At equilibrium, a high degree of adsorption (87%) was found with a low initial enzyme concentration (Fig. 6, exp. a). The degree of adsorption at equilibrium decreased to 73% with an initial enzyme concentration that is eight times higher (Fig. 6, exp. b).

Fig. 6 (exp. a) shows that a small part of xylanase enzyme, that was quickly

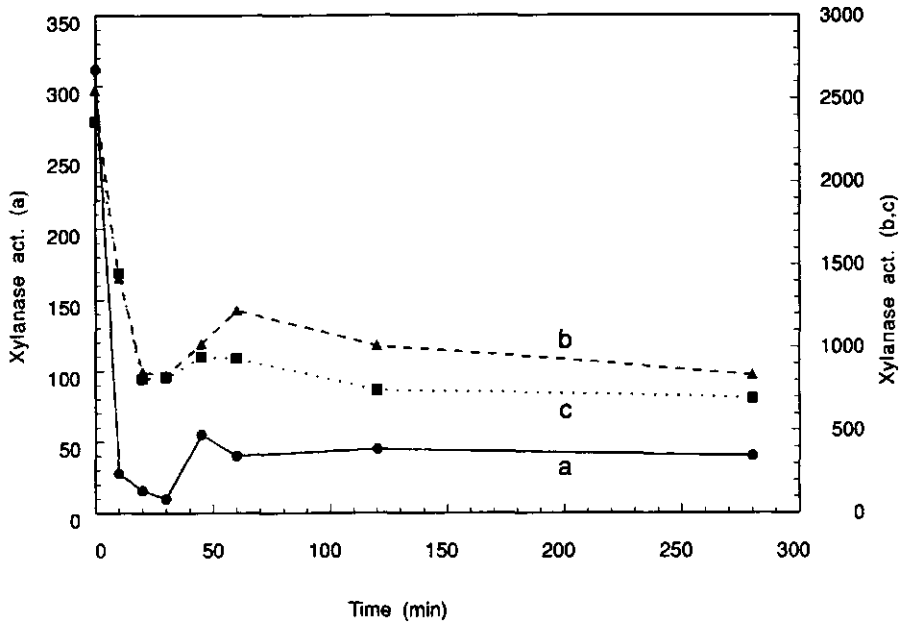

Fig. 6 Rate of the adsorption of xylanase from Pectinol by crosslinked xylan in 50 mM sodium acetate (pH 4.0) at 25 °C. In series c) degraded crosslinked xylan was used as an adsorbent. The starting enzyme concentration was 125 µg/ml for series a) ●—● , and 1 mg/ml for series b) ▲-▲ and c) ■··■ . Enzyme concentrations in supernatant are shown (mU/ml).

adsorbed in the beginning of the incubation, is released after 30 min incubation. However, adsorption continues and after 60 min about the same enzyme activity was measured in the supernatant as the enzyme activity found at equilibrium (20 h). It was expected that this sudden release of adsorbed xylanase was due to adsorbent degradation.

In experiment b there is also a decrease and after 30 min of incubation a subsequent increase of enzyme activity measured in the supernatant. The degree of adsorption after 20 h is higher than the degree of adsorption after 30 min. Both values are higher compared with the corresponding values from experiment a which might be due to normal kinetic effects.

The experiments were repeated with an independently prepared crosslinked xylan. Similar adsorption rates and adsorbent degradation were measured (results not shown).

The question remains whether adsorbent degradation is responsible for the

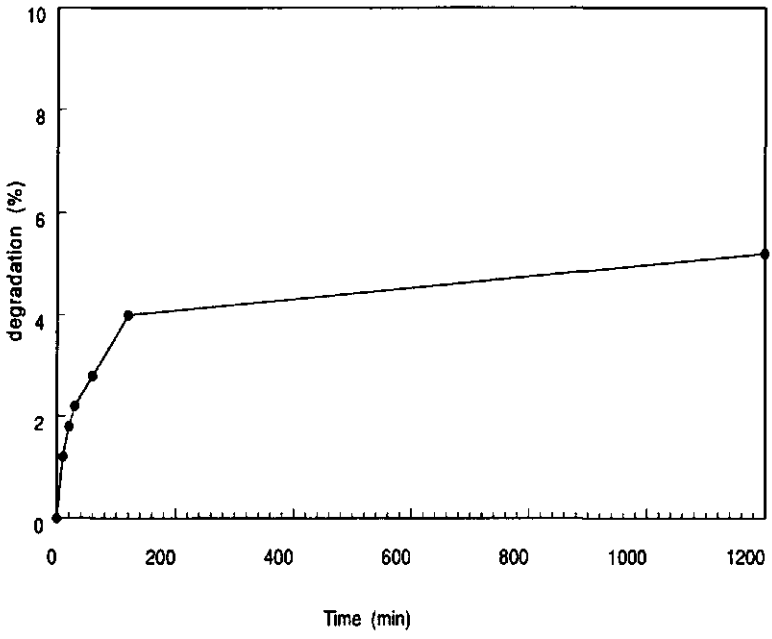

Fig. 7 Rate of degradation of crosslinked xylan (10 mg/ml) incubated with 1 mg/ml Pectinol in 50 mM sodium acetate (pH 4.0.).

changes in adsorbent-enzyme interaction. The normal asymptotic rate-curve expected for the degradation of the adsorbent (Fig. 7) is no positive indication in that matter. Therefore the adsorption rate experiments were repeated with an adsorbent already degraded. The same effect, a sudden gradual decrease in the degree of adsorption after 30 min and a subsequent gradual increase in the degree of adsorption thereafter, was found, although in this case the effect was less pronounced (Fig. 6, exp. c). However, no further degradation of the degraded crosslinked xylan was measured now. Thus, adsorbent degradation does not seem to be the correct explanation for the release of xylanase from the adsorbent after 30 min of incubation as will be further discussed below.

Capacity and adsorption equilibrium constant

Batch adsorption experiments with crude and purified Pectinol A1 were conducted to estimate the capacity of the crosslinked xylan adsorbent. To study adsorption at equilibrium the initial enzyme concentration was varied from 0.5 to 4 U/ml. Straight identical lines were found for crude and purified Pectinol if the reciprocal of the

bound enzyme activity was plotted against the reciprocal of the enzyme activity that remained in the supernatant (Rozie *et al.*, 1991). The capacity of crosslinked xylan for Pectinol xylanase was calculated from the intercept of the line at the ordinate (1.5 mg xylanase/g adsorbent). From the slope of the line an equilibrium adsorption constant was calculated of 4 x 10^6 L/mole assuming that 29 kD is the apparent molecular mass of the xylanase. K_a is 7 x 10^6 however if a molecular mass of 51 kD is assumed.

Discussion

Endo-xylanases make up only a small part of some commercial polysaccharide degrading enzymates. In this Chapter the isolation of xylanases from complex enzyme mixtures with crosslinked xylan as an affinity adsorbent is studied.

Our investigations revealed that two of the studied fungal enzymates, Pectinol A1 and Rohament CT (both from Röhm GmbH) are very similar if not identical although the area of application is different. Rohament CT is sold for its cellulolytic activity whereas Pectinol A1 is recommended for its pectinolytic activity. In both enzymates we found a major fraction of cellulase activity (results not shown) and a minor fraction of polygalacturonase activity. These enzymes were not adsorbed by crosslinked xylan. The xylanase present in these enzymates (one peak on FPLC) was isolated very well with a crosslinked xylan column. The specific acitivity rose from 5.5 to 160 U/mg. Analysis by SDS-PAGE showed two enzymes in the purified fraction (29 kD and 51 kD). Equilibrium experiments revealed that K_a is high (4 x 10^6 L/mole), which indicates that the xylanase is bound strongly to the adsorbent under the adsorption conditions used. However, the calculated capacity of crosslinked xylan for this specific xylanase is rather low (1.5 mg xylanase/g adsorbent) compared to a similar enzyme-adsorbent interaction as the α-amylase/crosslinked starch system in which case capactities as high as 185 mg enzyme/g adsorbent have been found.

Adsorption equilibrium between Pectinol xylanase and crosslinked xylan is reached within 2 h. A sudden release of xylanase activity after 30 min of incubation is consistently measured and this is not due to adsorbent degradation. The existence of competing enzymes with a slower initial adsorption rate compared to xylanase could be an explanation for this phenomenon since there is at least one enzyme in Pectinol

with no xylanase activity adsorbed by crosslinked xylan. This is shown by SDS-PAGE and FPLC. Another explanation for the sudden release of xylanase and adsorption thereafter, could be a rearrangement of the polysaccharide three-dimensional structure due to xylanase adsorption.

The other enzyme preparations used (Pectinex and KPB) are more complex and contain more side activities. In a former paper (Rozie *et al.*, 1992) we studied the adsorption properties of crosslinked xylan to some of these side activities in KPB enzymate. We found that ß-D-xylosidase, α-L-arabinofuranosidase and ß-D-galactosidase activities did not adsorb significantly to the crosslinked xylan adsorbent. There was also no adsorption for endo activities such as cellulase, ß-(1,3/6)-D-galactanase and ß-(1,4)-D-galactanase. Only polygalacturonase and xylanase activities were adsorbed by crosslinked xylan. However, in the complex enzyme mixtures KPB and Pectinex there is also a fraction of polygalacturonase and xylanase activity that is not adsorbed by crosslinked xylan (as is shown evidently in this Chapter). Xylanase and polygalacturonase adsorbed on crosslinked xylan simultaneously and appear in the same fraction. This is not only the case in the results presented, in which a sudden increase of ionic strength is applied for desorption but also in studies in which a NaCl gradient is applied (results not shown). The enzyme fractions desorbed from crosslinked xylan were further analysed by cation exchange chromatography. Both Pectinex and KPB contains a protein with an apparent molecular mass of 36 kD.

To start from less complex enzymates KPB was separated on DEAE-Biogel A into four xylanase containing fractions as described previously (Rozie *et al.*, 1992). These fractions were also chromatographed on crosslinked xylan and/or by cation exchange chromatography on Mono-S. From the fact that xylanase activity in enzymate fraction I is not adsorbed by the anion exchange material but is adsorbed by both cation exchange material and crosslinked xylan one is tempted to conclude that the latter adsorbent is only acting as a simple cation exchanger due to the glucuronic acid residues in the xylan backbone (6%). However, some observations are in contradiction with this hypothesis. In Fig. 5 for instance it is shown that some proteins in Pectinol A1, that were adsorbed by Mono-S, are not adsorbed by crosslinked xylan. From Figs. 2 and 3 it is also clear that some xylanases are adsorbed by Mono-S but not by crosslinked xylan. The latter adsorbent was reduced with sodium borohyride to discriminate between adsorption properties based on molecular recognition of the xylan backbone and adsorption based on the carboxylate groups in the biopolymer.

Although only part of the carboxylic acid residues were reduced (40%) this had a considerable effect on polygalacturonase adsorption but no effect on xylanase adsorption from KPB, which indicates that the negative charges do play an important role in polygalacturonase adsorption but are of no importance in xylanase adsorption. It seems that a crosslinked xylan which is chemically fully reduced or enzymatically stripped from its D-glucuronic acid residues (Johnson *et al.*, 1989) binds endo-xylanase much more specifically than polygalacturonase. The application of other starting materials by crosslinking homoxylans that can be isolated from esparto grass (Chanda *et al.*, 1950) or tobacco (Eda *et al.*, 1976) is a further possibility to improve xylanase adsorption specificity.

Crosslinked xylan is a suitable tool to purify xylanases from polysaccharide degrading enzyme mixtures, especially in the case in which the accompanying polygalacturonases do not adsorb to crosslinked xylan (Pectinol A1/Rohament CT). However, the adsorption capacity of crosslinked xylan for endo-xylanases at 22 °C is rather low which is a disadvantage when commercial applications are considered. Further optimization of the adsorption conditions may be performed by increasing the temperature (Pectinol A1 is stable upto 45 °C) and determining the optimal pH.

It is not clear why some endo-xylanases are adsorbed by crosslinked xylan and other endo-xylanases are not. In both cases the active site should provide binding properties to a non-crosslinked xylan backbone. Dekker & Richards (1976) mention some fungal xylanases to be glyco-proteins. This might influence the adsorption behaviour of the different enzymes. However, another more obvious explanation is that non-adsorbing endo-xylanases are hindered by substituents on specific positions of the modified polysaccharide residue while enzymes which adsorb are much less hindered by those modifications. To verify this hypothesis one needs to study in more detail the substrate specificity of the various enzymes, which could be done by studying break-down products from substrates differing in substituents and degree of substitution.

Acknowledgements

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Chapter 7

General Discussion

The development of three adsorbents for three polysaccharide splitting enzyme systems is described in the former chapters. Polygalacturonases and α-amylases are well known for their commercial applications and can be characterized as bulk enzymes. Xylanases are less known in this respect but the demand for enzyme systems that will convert waste material into useful products is growing rapidly. As mentioned in Chapter 1 the aim of this work was to find economically favourable purification methods for these enzymes based on molecular recognition. Starting point for this thesis was that the basic materials used for the preparation of the adsorbents had to be cheap substrates or cheap substrate analogs.

Adsorbent stability towards the enzyme mixtures

Alginate was thought to be a substrate analog for polygalacturonate since the L-guluronic acid blocks in this polymer have a similar three-dimensional structure. Thus, in preparing a suitable adsorbent for endo-polygalacturonases it seemed that the only condition that had to be fulfilled was keeping the alginate chain accessible to the enzyme and insoluble in the adsorbate, since enzymatic degradation of the adsorbent was not possible in this case. Insoluble uniform calcium alginate beads were easily prepared by dropping a sodium alginate solution into a solution of calcium chloride. The adsorption characteristics are described in Chapter 2 and seem promising with regard to commercial fluid bed applications (Somers et al., 1989).

In contrast with this substrate analog, the adsorbents prepared from natural substrates such as starch and xylan appeared to be degradable. However, crosslinked xylan which is suitable for adsorption is modified that much that degradation of this adsorbent is limited to approximately 4% of the mass of the adsorbent (Chapter 5). Further degradation does not occur, even if the adsorbent is reused for adsorption. The latter is not the case with crosslinked starch. Although potato starch can be crosslinked up to a degree that degradation does not occur, adsorption of α-amylase on that material is no longer possible. Crosslinked starch that is suitable for adsorption is also degraded more or less by α-amylases. Although this degradation process appears to continue and does not stop at any level, it can be limited by using short adsorption and desorption time spans. In this respect it is also an advantage that

adsorption is maximum at lower temperatures (4 °C) with a low degradation velocity. Degradation at higher temperatures under desorption conditions (up to 70 °C) can be prevented largely by the use of glycerol in the desorption buffer. However, due to slight degradation it is still a problem in adsorption studies to discriminate between the adsorption process and enzyme catalysis as is mentioned recently by Leloup et al. (1991) in their study on α-amylase adsorption on starch crystallites.

Interactions between enzymes and adsorbents

The polysaccharides that are used for the preparation of the adsorbents are different with respect to the residues from which they are built up. Starch and xylan are polysaccharides with a main chain composed of 1,4-ß-glycosidically linked glucose and xylose, respectively. Polygalacturonate and alginate consist of residues with a carboxylic acid group. The heteropolymer xylan does have a backbone made up from neutral xylose residues but the side chains contain amongst others D-glucuronic acid. From the polysaccharides used in this study only the homopolymer starch does not contain carboxylic acid groups, but it does contain phosphate groups that may act as weak cation exchangers. However, the phosphorus content of potato starch is only about 0.04 % (Greenwood & Thomson, 1962) and no phosphate esters are expected in crosslinked starch since the ester groups are hydrolyzed during the reaction of starch with epichlorohydrin in an alkaline solution.

It is described in Chapter 2 that the affinity of endo-polygalacturonase towards calcium alginate was probably not due to biospecific molecular recognition that was expected from the similarity between D-guluronic and D-galacturonic acid residues, but primarily to ionic interactions between polygalacturonase and a charged polymer. Similar ionic interactions are an explanation for the adsorption of endo-polygalacturonases to crosslinked xylan. In Chapter 6 it is described that the affinity of endo-xylanases towards crosslinked xylan may be due to biospecific molecular recognition. The latter is certainly the case in the α-amylase/crosslinked starch adsorption system in which ionic interactions play a minor role. Van der Waals forces are probably responsible for the interaction between α-amylase and starch as was concluded from the large effect of temperature on the adsorption: at higher temperatures, less enzyme appeared to bind to the adsorbent.

In the enzyme-adsorbent systems we found also corresponding enzyme activities which did not bind to the adsorbents: endo-polygalacturonases and endo-xylanases that

did not adsorb to calcium alginate and crosslinked xylan, respectively. On crosslinked starch, no adsorption of fungal α-amylases such as that from *Aspergillus oryzae* were observed. It is not entirely clear why some endo-enzymes are adsorbed by these adsorbents and other similar enzymes are not. As is mentioned in Chapter 6, a possible explanation is that the non-adsorbing enzymes are glyco-enzymes (Dekker & Richards, 1976). Another, more obvious, explanation is that non-adsorbing polysaccharide degrading enzymes are hindered by substituents on specific positions of the modified polysaccharide while enzymes which adsorb are much less hindered.

Polysaccharidases that have catalytic activity for a certain substrate can have binding properties towards another substrate. An example thereof is the presence of a cellulose-binding domain which is distinct from the active site in a specific xylanase of *Pseudomonas fluorescens* (Ferreira et al., 1990). Generally this means that in some cases adsorption of substrate-odd enzymes on modified substrates may be due to biospecificity rather than merely accidental electrostatic or hydrophobic effects.

Equilibrium adsorption studies

Equilibrium adsorption constants and enzyme capacities of the adsorbents were calculated from equilibrium experiments. Equilibrium was reached slowly (about 48 h) with the rather large alginate beads as an adsorbent. The same time span was initially used for adsorption on crosslinked starch, but the adsorption times were considerably shortened by an enzymatic or mechanical pretreatment of the adsorbent (Somers, 1992). With crosslinked xylan equilibrium was reached within 2 h. The calculated equilibrium constants of the enzyme- adsorbent systems are shown in Table 1 and are in the range between $0.5 * 10^5$ and $4 * 10^6$ L/mole. These values are rather low but still in the normal range for affinity systems (K_a between 10^5 and 10^{12} L/mole), indicating that the enzyme is bound firmly enough under optimal adsorption conditions but is rapidly desorbed when an essential parameter such as ionic strength or temperature is changed. The calculated capacity of the adsorbent is very large for crosslinked starch and rather low for crosslinked xylan. The capacity of wet calcium alginate for endo-polygalacturonase is about 25 mg/g which complies with about 80 mg/g dry adsorbent (Table 1). In the latter system the calculated data (capacity and K_a) are based on equilibrium experiments with an enzyme mixture while the calculations are made with the specific activity and the molecular weight of one particular polygalacturonase. In the commercial enzyme preparation (Rapidase) the

Table 1
Calculated adsorption equilibrium constants and capacities of the enzyme/adsorbent systems studied.

Enzyme/adsorbent system	Source	K_a (L/mole)	Capacity (mg enzyme/ g adsorbent)
Endo-polygalacturonase/ calcium alginate	Rapidase	$6.1 * 10^5$	80
α-Amylase/ crosslinked starch	Maxamyl	$3.8 * 10^5$	185
	Dexlo	$0.53 * 10^5$	71
Endo-xylanase/ crosslinked xylan	Pectinol	$4 * 10^6$	1.5

two most abundant endo-polygalacturonases show affinity for calcium alginate beads. The calculations in Chapter 2 are based on a specific activity of 2750 U/mg and a molecular weight of 38 kD of an isolated endo-polygalacturonase (endoPG II). However, at that time, a misunderstanding about the identity of the major enzyme that adsorbed on calcium alginate beads led to the erroneous use of these values for specific activity and molecular weight. Later investigations (Kester & Visser, 1990) showed that the specific activity and molecular weight for the main enzyme (endoPG I) in a prepurified enzyme mixture that adsorbed on calcium alginate beads are 550 U/mg and 55 kD, respectively. The adsorption capacity and K_a that correspond with these data are shown in Table 1.

In conclusion, the capacities of crosslinked starch and calcium alginate, shown in Table 1, are large enough to consider commercial applications of these adsorbents. For crosslinked xylan however, only laboratory applications are to be expected.

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Summary

The aim of this work was to find economically favourable, affinity based, purification methods for several polysaccharide splitting bulk enzymes. The framework in which this study is done is described in Chapter 1.

Chapter 2 describes the adsorption of endo-polygalacturonase (endoPG) from a commercial enzyme preparation (Rapidase) to calcium alginate beads. Approximately 75% of the various polygalacturonase activities from Rapidase can be adsorbed at pH 4.4 by calcium alginate beads as well as by crosslinked sodium alginate powder. Equilibrium experiments were conducted to determine a parameter (k) that represents the degree of interaction between endoPG and the adsorbent. This parameter can be influenced by a change in pH and ionic strength of the adsorbate. At pH 3.8 the degree of interaction is 20 times larger than at pH 4.2. There is increased adsorption when the ionic strength is lowered, but a small amount of $CaCl_2$ is required to keep the calcium alginate beads stable.

Despite the resemblance in structure between L-guluronate blocks and polygalacturonate, a lower k value was found when the alginate, used for the preparation of the beads, contained a larger proportion of guluronic acid residues. There is no evidence that L-guluronic blocks in the alginate chain are responsible for the large affinity of endo-PG to this adsorbent. The influence of the pH and the ionic strength and the lack of endoPG inhibition by sodium alginate are indicative for ionic interactions between endoPG and the alginate chains.

Ionic interactions were of no importance in the interaction between α-amylase and crosslinked starch as is described in the chapters 3 and 4. Crosslinked potato starch was prepared as an affinity adsorbent for bacterial α-amylase. To this end, reaction parameters for crosslinking in an ethanol/water solvent were investigated (Chapter 3). The degree of crosslinking, and consequently the suitability of crosslinked starch as an adsorbent for α-amylase, changed by altering these parameters. An increase in the degree of crosslinking of the adsorbent caused lower affinity for bacterial α-amylase which resulted in an unfavourable decrease in adsorption capacity and a favourable decrease of degradation of the adsorbent by the enzyme.

The adsorption and desorption characteristics of two bacterial α-amylases (*B.subtilis*, *B.licheniformis*) on crosslinked potato starch are described in Chapter 4. A capacity of about 185 mg (*B.subtilis*) and 71 mg (*B.licheniformis*) protein per g

adsorbent can be realized. However, at 4 °C a smaller adsorption constant (K_a) was measured for the enzyme from *B.subtilis* (0.53 * 10^5 L/mole) than for the *B.licheniformis* enzyme (3.8 * 10^5 L/mole). The K_a decreases with increasing temperature suggesting that association is caused by van der Waals forces. Comparison of the adsorption of the α-amylases to crosslinked starch with the activity of the enzymes on their natural substrate reveals that the velocity constant of the backward reaction of the enzyme-adsorbent complex increases strongly with increasing temperatures (*B.subtilis* α-amylase, k_2 (20 °C)/ k_2 (4 °C) ≈ 30). Desorption can be accomplished by a raise in temperature. Glycerol (20%) is added to the desorption buffer to stabilize the enzymes and protect the adsorbent against enzymic attack. The optimal desorption temperature for the *B.subtilis* enzyme is 60 °C. For the *B.licheniformis* enzyme this value is 70 °C or even higher. The adsorption velocity of α-amylases to freshly crosslinked starch is low due to the low accessibility of the adsorbent. This can easily be improved by enzymatic modification. Thus, bacterial α-amylases can be adsorbed and desorbed within short time spans (10 min) in sufficiently high amounts to make such an affinity purification process economically feasible.

In order to facilitate the purification of xylanases from *Aspergillus niger*, an affinity adsorbent has been developed from oat spelts xylan (Chapter 5). A suitable adsorbent was only obtained by crosslinking oat spelts xylan with epichlorohydrin in water but not in ethanol or ethanol water mixtures. After some initial degradation of the adsorbent (approx. 4%), no further biodegradation was measured with a reused adsorbent. Up to 60% of the xylanase activity from an *Aspergillus niger* enzyme mixture (50 mU/ml) was adsorbed at pH 4 The degree of adsorption to crosslinked xylan of four fractions of this preparation, previously separated by DEAE-Biogel A chromatography, varied between 40 and 90%.

Adsorption was strongly dependent on pH and ionic strength and desorption was easily accomplished by an increase in ionic strength. In addition to xylanases, polygalacturonases also adsorbed to the matrix probably due to the D-glucuronic acid moieties in xylan. No significant adsorption of ß-D-xylosidase, α-L-arabinofuranosidase, ß-D-galactosidase, ß-(1,4)-galactanase, ß-(1-3/6)-D-galactanase or cellulase activities was found.

The binding behaviour of four commercial fungal enzyme preparations on crosslinked xylan is presented in Chapter 6. The xylanase activity in Pectinol A1

(Röhm GmbH, Darmstadt, Germany) was efficiently purified with crosslinked xylan. The specific endo-xylanase activity increased from 5.5 U/mg up to 160 U/mg. Two proteins were found with SDS-PAGE in purified Pectinol (29 and 51 kD) whereas a K_m of 1.1 mg/ml was measured. Equilibrium adsorption studies revealed a rather low capacity for the Pectinol endo-xylanase (1.5 mg xylanase/g adsorbent). The calculated K_a was $4 * 10^6$ L/mole.

Some endo-xylanases were also adsorbed by cation exchange material. However, from crosslinked xylan chromatography and additional FPLC studies it appeared that the adsorption properties of crosslinked xylan were not only due to the cation-binding properties of this adsorbent.

Chapter 7 is an evaluation of the foregoing chapters. The adsorption properties of three kinds of economically important polysaccharide splitting enzymes are studied in this work. A cheap substrate analog and crosslinked substrates were used as adsorbents. The magnitude of the capacities of calcium alginate and crosslinked starch towards endo-polygalacturonases and α-amylases, respectively, is such that commercial applications can be considered. Only laboratory applications are foreseen for crosslinked xylan as affinity adsorbent for specific endo-xylanases since the capacity of this adsorbent is rather low.

Samenvatting

Het vinden van commercieel toepasbare zuiveringsmethodieken voor bepaalde polysaccharide splitsende enzymen gebaseerd op affiniteits-interacties was het doel van het werk voor dit proefschrift. Dit doel en het kader waarin dit onderzoek is gedaan staan beschreven in Hoofdstuk 1.

Endo-polygalacturonase is een enzym dat bijvoorbeeld bij het vervloeien van landbouwprodukten en bij het klaren van vruchtensappen wordt gebruikt. In Hoofdstuk 2 wordt de adsorptie van endo-polygalacturonase (endoPG) vanuit een commercieel te verkrijgen enzymmengsel (Rapidase) op calciumalginaat-bolletjes beschreven. Ongeveer 75% van de polygalacturonase activiteit van Rapidase kan bij pH 4,4 worden geadsorbeerd; enkele van de multipele endoPG's worden niet gebonden. Er is een parameter (k), die de mate van interactie tussen endoPG en het adsorbens weergeeft, bepaald door het uitvoeren van evenwichts-experimenten. De grootte van deze parameter kan worden beïnvloed door de pH dan wel de ionsterkte van de te gebruiken buffer te veranderen. Bij pH 3,8 is de mate van interactie 20 maal zo groot als bij pH 4,2. Optimale adsorptie werd gevonden bij een zo laag mogelijke ionsterkte, hoewel een kleine hoeveelheid $CaCl_2$ nodig blijft om de calciumalginaat-bolletjes stabiel te houden.

Een kleinere k werd gevonden wanneer als bron voor de calciumalginaat-bolletjes een alginaat werd gebruikt dat een groot gehalte L-guluronzuur bevatte. Dit is tegengesteld aan hetgeen werd verwacht omdat er qua structuur een zekere gelijkenis bestaat tussen polygalacturonzuur en de L-guluronzuur-blokken in het alginaat. Tot op heden is er dan ook geen bewijs dat de L-guluronzuur-blokken in het alginaat verantwoordelijk zijn voor de waargenomen hoge affiniteit van endoPG ten opzichte van dit adsorbens. De wijze waarop pH en ionsterkte de adsorptie beïnvloeden gevoegd bij het feit dat geen inhibitie wordt gevonden van de polygalacturonase activiteit door natriumalginaat zijn aanwijzingen dat hier sprake is van normale niet-biospecifieke ionogene interacties tussen endoPG en het alginaatpolymeer. Ionogene bindingen spelen geen rol in de interactie tussen α-amylase en verknoopt zetmeel, hetgeen wordt beschreven in de hoofdstukken 3 en 4. α-Amylase is een industriëel belangrijk endo-enzym dat gebruikt wordt om zetmeel af te breken. Er is verknoopt aardappelzetmeel gesynthetiseerd als een affiniteitsadsorbens voor bacteriëel α-amylase. Voorts zijn de reactie-parameters, voor wat betreft de verknoping met

epichloorhydrine in een ethanol/water mengsel, bestudeerd (Hoofdstuk 3). De verknopingsgraad en daarmee de mate van geschiktheid om verknoopt zetmeel als affiniteitsadsorbens te gebruiken, veranderde door het aanpassen van de reactie-parameters. Een grotere verknopingsgraad veroorzaakte minder affiniteit voor bacteriëel α-amylase, hetgeen resulteerde in een ongunstige verlaging van de adsorptie-capaciteit en een gunstige verlaging van de mate van afbraak van het adsorbens door het enzym.

In Hoofdstuk 4 zijn adsorptie- en desorptiekarakteristieken van twee bacteriële α-amylases (*B.subtilis*, *B.licheniformis*) op verknoopt aardappelzetmeel beschreven. Voor α-amylase van *B.subtilis* kan een capaciteit van 185 mg eiwit en voor *B.licheniformis* een capaciteit van 71 mg eiwit per gram adsorbent worden gerealiseerd. De gemeten adsorptie-constante (K_a) met *B.subtilis* enzym is echter bij 4 °C kleiner dan die met *B.licheniformis* α-amylase. De (K_a) daalt bij toenemende temperatuur, hetgeen aangeeft dat de interactie wordt veroorzaakt door Van der Waals krachten. Wanneer de adsorptie van α-amylase op verknoopt aardappelzetmeel wordt vergeleken met de normale enzym-activiteit met aardappelzetmeel als substraat blijkt dat de snelheidsconstante van de teruggaande reactie, d.w.z. het uiteenvallen van het enzym-adsorbens complex, vrij snel groter wordt bij toenemende temperatuur. Bij *B.subtilis* α-amylase is deze k_2 bij 20 °C ten opzichte van die bij 4 °C een factor 30 hoger. Vrijwel volledige desorptie kan worden bewerkstelligd door een verhoging van de temperatuur. Glycerol (20%) werd aan de desorptie-buffer toegevoegd om de enzymen te stabiliseren en het adsorbens tegen enzymatische afbraak te beschermen. Voor *B.subtilis* α-amylase ligt de optimale desorptie-temperatuur op 60 °C, bij het thermostabiele *B.licheniformis* α-amylase op 70 °C. De snelheid van de adsorptie van α-amylase op verknoopt zetmeel is in het begin laag, hetgeen te wijten is aan de op dat moment lage toegankelijkheid van het adsorbens. Dit kan vrij gemakkelijk worden verbeterd door bijvoorbeeld een voorafgaande behandeling van het adsorbens met α-amylase. Korte adsorptie- en desorptie-tijden van ca. 10 minuten kunnen op deze manier worden gerealiseerd. De korte cyclustijden en de relatief grote capaciteit van het adsorbens lijken een commerciële toepassing mogelijk te maken.

Endo-xylanases worden gebruikt in de papier- en in de voedingsmiddelenindustrie. Op soortgelijke wijze als voor α-amylase is voor xylanases, afkomstig van *Aspergillus niger*, een adsorbens ontwikkeld met xylaan als basismateriaal. Dit wordt beschreven in Hoofdstuk 5. Verknoping van xylaan met epichloorhydrine in water, en dus niet in

een ethanol/water-mengsel, leverde een geschikt adsorbens op. Na een initiële afbraak van het adsorbens (ca. 4%) kon geen verdere afbraak meer worden vastgesteld wanneer het adsorbens werd hergebruikt. Ca. 60% van de xylanase activiteit van een *Aspergillus niger* enzymmengsel (50 U/ml) werd bij pH 4 geadsorbeerd. Een voorafgaande scheiding van dit enzymmengsel met DEAE-Biogel A chromatografie resulteerde in 4 fracties. De mate van adsorptie op verknoopt xylaan van deze fracties varieerde van 40 tot 90%.

De adsorptie was afhankelijk van de pH en de ionsterkte en complete desorptie werd dan ook bewerkstelligd door een verhoging van de ionsterkte. Behalve xylanases adsorbeerden ook polygalacturonases aan het adsorbens, hetgeen kan worden verklaard met de aanwezigheid van D-glucuronzuur eenheden in het uitgangsmateriaal xylaan. Significante adsorptie van nevenactiviteiten als ß-D-xylosidase, α-L-arabinofuranosidase,ß-D-galactosidase,ß-D(1,4)-galactanase,ß-(1,3/6)-D-galactanase en cellulase activiteiten werd niet gevonden.

Hoe vier commerciële schimmel-enzymmengsels zich gedragen met verknoopt xylaan als adsorbens staat beschreven in Hoofdstuk 6. Endo-xylanase in Pectinol A1 (Röhm GmbH, Darmstadt, Duitsland) kon met verknoopt xylaan verregaand gezuiverd worden. De specifieke endo-xylanase activiteit werd verhoogd van 5,5 U/mg tot 160 U/mg. In gezuiverd Pectinol werden met SDS-PAGE twee eiwitten gevonden (29 en 51 kD), terwijl een K_m van 1,1 mg/ml is gemeten. De adsorptieconstante van dit endo-xylanase was 4 * 10^6 L/mol, maar de capaciteit van het adsorbens was relatief laag: 1,5 mg xylanase/ g adsorbens.

Enkele endo-xylanases adsorbeerden ook op een kationen-wisselaar. Gezien de resultaten met chromatografie op verknoopt xylaan aangevuld met FPLC-onderzoek lijkt het echter zo te zijn dat de adsorptie-eigenschappen van verknoopt xylaan niet alleen verklaard kunnen worden door de aanwezige positieve lading op het adsorbens.

Hoofdstuk 7 is een beknopte evaluatie van het voorafgaande werk. De adsorptie-eigenschappen van drie commerciëel belangrijke polysaccharide splitsende enzymen worden vergeleken waarbij goedkope substraten-analogen en verknoopte substraten zijn gebruikt als adsorbentia. De capaciteiten van calciumalginaat en verknoopt zetmeel zijn qua grootte zodanig dat een commerciële toepassing tot de mogelijkheden behoort. Aangezien de capaciteit van verknoopt xylaan voor specifieke endo-xylanases relatief laag is moet het toepassingsgebied voor dit adsorbens vooral in de laboratoriumsfeer worden gezocht.

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 24 juli 1959 te Nijmegen. In 1977 behaalde hij het diploma V.W.O.-B aan het Dominicus College te Nijmegen. In hetzelfde jaar begon hij met de scheikunde-studie aan de Katholieke Universiteit Nijmegen. Na het behalen van het kandidaatsdiploma in 1980 werden het hoofdvak organische chemie (Prof. G.I. Tesser) en de bijvakken chemische microbiologie en vakdidaktiek gekozen. Na het doctoraalexamen in 1985 trad hij in dienst bij de Stichting Technische Wetenschappen (S.T.W./N.W.O.). Het promotie-onderzoek dat heeft geleid tot dit proefschrift vond plaats van 1985 tot in 1989 op het Laboratorium voor Levensmiddelentechnologie van de Landbouwuniversiteit te Wageningen, onder leiding van Dr. J. Visser, Prof. F.M. Rombouts en Prof. K. van 't Riet. Sinds 1 januari 1990 is hij werkzaam bij DSM Research te Geleen.