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# 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.*



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## 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 $\alpha$-amylase inhibitor from wheat kernel was used as a ligand for purification of insect $\alpha$-amylase (Buonocore *et al.*, 1975). Wheat $\alpha$-amylase was purified with immobilized $\beta$-cyclodextrin (Silvanovich & Hill, 1976) and immobilized glycogen (Tkachuk, 1975). Bacterial $\alpha$-amylase however, does not bind to the $\beta$-cyclodextrin. No suitable ligand-carrier adsorbents are known for the purification of bacterial and fungal $\alpha$-amylases although from an economic viewpoint the isolation of such amylases is quite important.

Another approach to the preparation of a suitable adsorbent for $\alpha$-amylase is to modify its substrate. Starch granules, especially those of potato, are only slowly degraded by $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-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 $\mu$l of the supernatant was added to 790 $\mu$l 0·1 M sodium acetate buffer (pH 5·0) and 10 $\mu$l 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**

$\alpha$-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 $\mu$l of an enzyme solution (0–0·5 U/ml). The mixture was incubated for 30 min at 30°C after which 200 $\mu$l was taken which was added to 800 $\mu$l 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 $\mu$mol 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 $\alpha$-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 $\alpha$-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

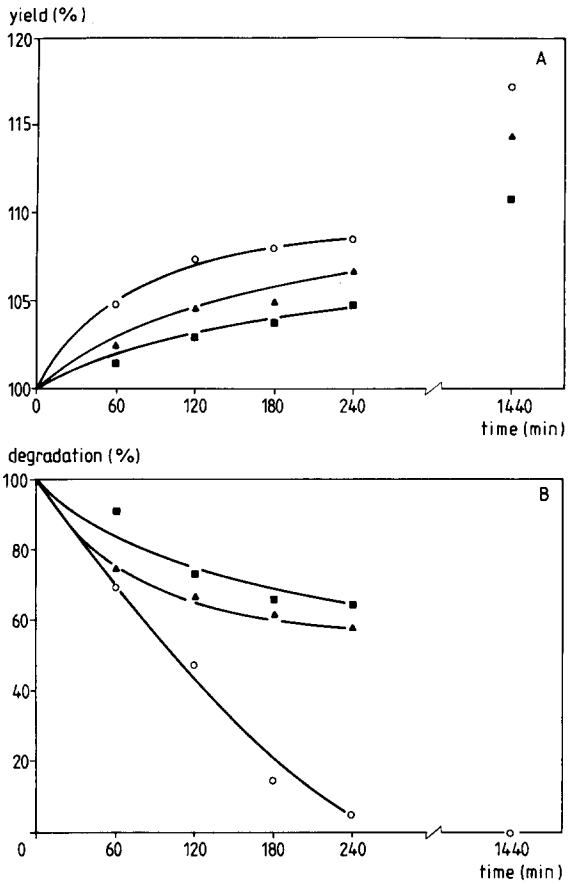


**Fig. 1.** The influence of reaction time and temperature on product yield of the crosslinking reaction (A) and on the degree of degradation of the product by α-amylase (B). Reaction conditions for crosslinking: amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0. Reaction 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.

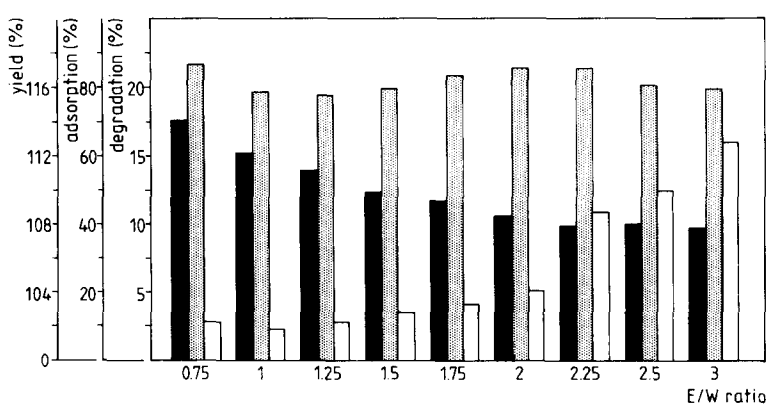


**Fig. 2.** Influence of the solvent composition in the crosslinking reaction on yield and properties of the adsorbent. Reaction conditions: amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; reaction temperature 45°C; reaction time 240 min. Solid bars, yield (%); hatched bars, adsorption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

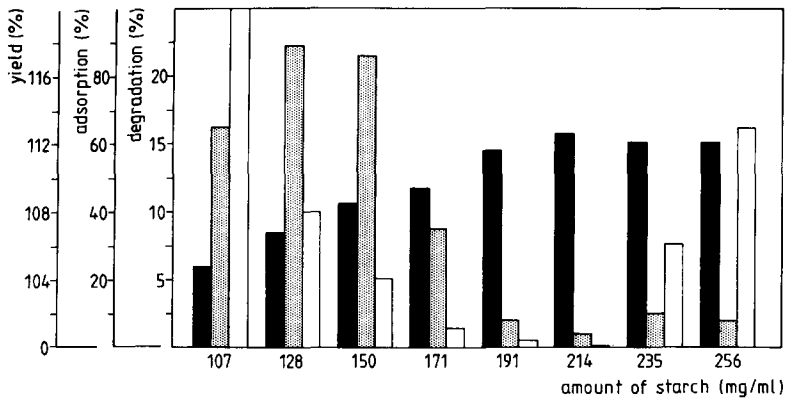


**Fig. 3.** Influence of the amount of starch used in the crosslinking reaction on yield and properties of the adsorbent. Reaction conditions: ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C; reaction time 240 min. Solid bars, yield (%); hatched bars, adsorption of $\alpha$-amylase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

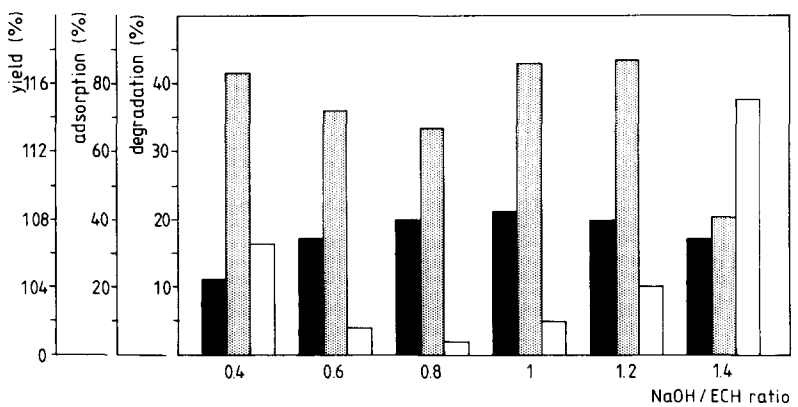


**Fig. 4.** Influence of the NaOH/ECH ratio in the crosslinking reaction on yield and properties of the adsorbent. Reaction conditions: amount of starch 150 mg/ml; ECH/GM ratio 0·65; E/W ratio 2·0; reaction temperature 45°C; reaction time 240 min. Solid bars, yield (%); hatched bars, adsorption of α-amylase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

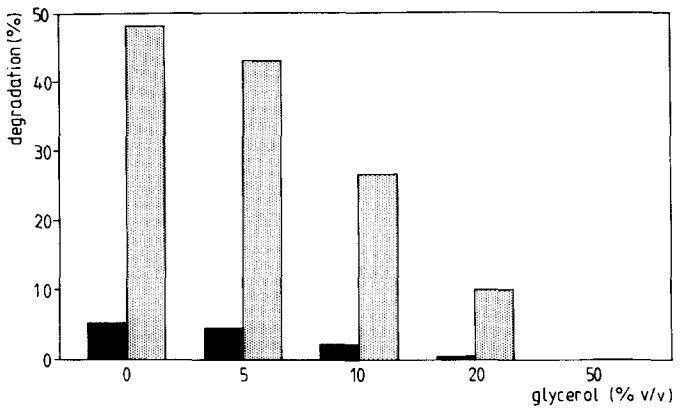


**Fig. 5.** The influence of glycerol on the degradation of crosslinked starch by α-amylase at 70°C. Reaction conditions for the synthesis of the adsorbents: amount of starch 150 mg/ml; ECH/GM ratio 0·65; NaOH/ECH ratio 1·0; E/W ratio 2·0; reaction temperature 45°C. Adsorbent A (solid bars), reaction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

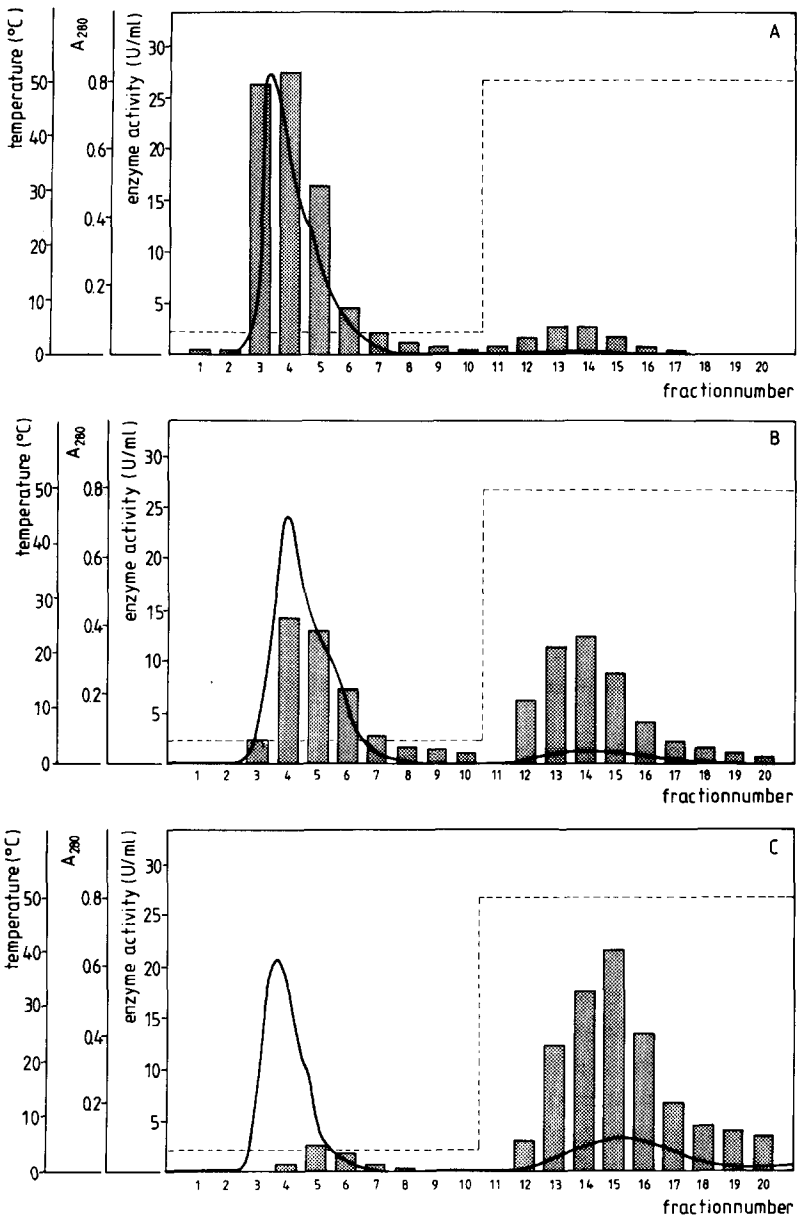


**Fig. 6.** Adsorption and desorption of bacterial $\alpha$-amylase from three different cross-linked starches. Hatched bars, $\alpha$-amylase activity (U/ml); ——, absorbance at 280 nm; – – –, temperature (°C). Reaction conditions for the synthesis of the adsorbents: (A) Amount of starch 150 mg/ml; ECH/GM 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.

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