ENTRAPMENT OF CHEMICAL DERIVATIVES OF GLYCOAMYLASE IN CALCIUM ALGINATE GELS

by

BIRTE SVENSSON and MARTIN OTTESEN

Department of Chemistry, Carlsberg Laboratory
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

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Glucoamylase was retained in calcium alginate gels, when the molecular weight of the enzyme was increased, e.g., by attachment to polymeric supports or by intermolecular cross-linking. Alternatively, the enzyme could be retained by covalent attachment to the alginate matrix. In the majority of the glucoamylase derivatives more than 50 % of the initial activity determined with maltose or partially hydrolyzed starch as substrate was recovered, while upon entrapment in gel particles, less than 40 % and 6 % of the initial activity towards maltose and partially hydrolyzed starch, respectively, was recovered. Thus, diffusional and steric limitations on substrate access seem to influence the apparent enzymic activity, in spite of the high permeability of calcium alginate gels. Dextran-coupled glucoamylase entrapped in calcium alginate gels has been used as a packed-bed reactor for continuous hydrolysis of oligodextrins during a period of two months with no change in activity.

1. INTRODUCTION

Continuous processing with immobilized microbial cells represents a new approach to biocatalysis. Thus, industrial production of L-malic acid (2), L-aspartic acid (22) and high fructose syrups (1) have already been performed by utilization of non-living immobilized microorganisms and interest is increasing in the application of viable immobilized cells in the accomplishment of multistep reactions. Examples of products obtained in a small scale by continuous processes using living immobilized cells are L-isoleucine (30), 2,3-butanediol (4),

citric acid (29), ethanol (8, 9), beer (32) and the antibiotics penicillin G (24) and bacitracin (18). The combined action of microorganisms and externally added enzymes is frequently utilized in industrial processes such as production of ethanol from biomass, where cellulases or amylolytic enzymes hydrolyse polysaccharides to fermentable sugars before conversion to ethanol by yeast strains (7). Reactors of coimmobilized microorganisms and enzymes are therefore of potential interest for continuous operation in such processes. The extremely mild conditions required for immobilization of living cells are

often fulfilled by entrapment in gel matrices of calcium alginate (3). However, due to the high permeability of such polysaccharide gels (12) most enzymes need to be converted to larger, retainable molecular species or, alternatively, the enzymes have to be covalently attached to the gel matrix material.

In the present work a series of chemically modified preparations of glucoamylase suitable for immobilization in calcium alginate gel particles was synthesized by intermolecular cross-linking, by attachment to soluble or insoluble polymers or by direct coupling to the alginate. The choice of glucoamylase was motivated by the wide application of this enzyme for saccharification in the manufacture of ethanol and in the brewing industry. Coimmobilization of glucoamylase and yeast utilized for the production of low calorie beer will be described in a subsequent paper.

2. MATERIALS

Commercial glucoamylase from *Aspergillus niger*, Amyloglucosidase AMG 200 L, and heat stable α -amylase from *Bacillus licheniformis*, Termamyl 60 L, were generous gifts of the Novo Industries, Bagsværd, Denmark. Glucoamylase was isolated in homogeneous form from amyloglucosidase AMG 200 L by a modification of procedures reported in the literature (14, 21, 28).

Sodium alginate (Protanal LF 10/60) was obtained from BDH Chemicals, Poole, GB, Dextran T 500 from Pharmacia Fine Chemicals, Uppsala, Sweden, aminoethylcellulose, Cellex-AE, from Bio-Rad, Richmond, Calif., dimethylsuberimide dihydrochloride from Sigma, St. Louis, Mo., 25 % aqueous solution of glutaraldehyde from Fluka, Buchs, G.F.R., 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride from Pierce, Rockford, Ill., 1,4-butanediol diglycidyl ether from EGA-Chemie, Steinheim, G.F.R., and glycyl-L-leucine amide hydrochloride from Bachem, Liestal, Switzerland. Maltose monohydrate, soluble starch and reagents for determination of glucose, System Glucose, comprising glucose dehydrogenase, mutarotase, and NAD were from Merck, Darmstadt, G.F.R.

3. METHODS

3.1. Calcium alginate gel particles

Spherical beads of precipitated alginate was prepared essentially as previously described (11, 12). A 3 % solution of sodium alginate in water was autoclaved at 120 °C for 1 hour and mixed with an appropriate amount of dissolved or suspended enzyme derivative. Spherical particles of approximately 2 mm diameter were obtained by dropwise addition from a needle of 0.9 mm orifice into an excess of 0.1 M-calcium chloride, 0.05 M in sodium acetate, pH 4.5. After at least two hours of curing, the beads were washed by several changes of this buffer. When alginate-coupled amyloglucosidase derivatives were used the particles were made in a similar manner right after coupling of enzyme or from a 2 % solution of alginate-glucoamylase freshly prepared by vigorous stirring at room temperature.

3.2. Polymerization of glucoamylase

3.2.1. Cross-linking by glutaraldehyde

Crude glucoamylase diluted three-fold and adjusted to a pH value in the range 6.0–9.0 was made 0.02–2 % in glutaraldehyde (17). After standing for 2.5 hours at room temperature sodium borohydride was slowly added to a final concentration of 0.1 M. The reaction mixtures were left for 20 minutes and then mixed in a ratio of 1:9 (v/v) with 3 % sodium alginate for immediate entrapment.

3.2.2. Cross-linking by dimethylsuberimide

Crude glucoamylase was adjusted to pH 8.0 and dialyzed against 0.25 M-triethanolamine pH 8.0 and made 0.004–0.18 M in dimethylsuberimide (17). After 1 hour at 4 °C the reaction was quenched by an excess of ammonium acetate, diluted with four volumes of 0.1 M-sodium acetate, pH 4.5, and then mixed in a ratio of 1:9 (v/v) with 3 % alginate for immediate entrapment.

3.3. Coupling of glucoamylase to polymeric supports

3.3.1. Coupling to dextran

A 1 % solution of Dextran T 500, $\bar{M}_n = 180,000$, was activated at pH 10.7 by two

portions of cyanogen bromide ($2.5 \text{ mg} \cdot \text{ml}^{-1}$ of dextran solution) and coupled to protein as described previously (15, 25). At the end of the activation step the solution became cloudy, pH was adjusted to 9.0 and the major part of cyanogen bromide was removed by dialysis for 2 hours at 4°C against 20 volumes of water adjusted to pH 9.0 with sodium carbonate. The pH of cyanogen bromide activated dextran was adjusted to the value of the coupling step and crude glucoamylase adjusted to the same pH was added. After standing for 20 hours at 4°C excess glycine was added in order to destroy remaining reactive groups and 6 hours later the coupling mixture was dialyzed against 0.05 M-sodium acetate, pH 4.5. After 16 hours dialysis the product was lyophilized.

3.3.2. *Coupling to aminoethylcellulose*

Aminoethylcellulose (5 g, $0.37 \text{ meq} \cdot \text{g}^{-1}$) was pretreated as described by GLASSMEYER and OGLE (10), suspended in 30 ml of 1 M-sodium phosphate, pH 7.0, and stirred with 20 ml of 25% glutaraldehyde for 2 hours at room temperature. Excess reagent was removed by suction filtration and washing with 1 M-sodium phosphate, pH 7.0. Crude glucoamylase, diluted 4–30 fold with 1 M-sodium phosphate, pH 7.0, was mixed with activated resin in a ratio of 5:1 (v/wt) and left for 2 hours at room temperature. The products were washed by 1 M-sodium phosphate, pH 7.0, followed by 0.05 M-sodium acetate, pH 4.5, and treated for 2 hours at room temperature with 0.1 M-sodium borohydride. The washing was continued with the acetate buffer until no further release of active enzyme could be detected.

3.3.3. *Coupling to oxirane-alginate*

Sodium alginate was activated by bisepoxide essentially as described by SUNDBERG and PORATH for other polysaccharides (23). A 2% solution of alginate in 0.3 M-sodium hydroxide containing $2 \text{ mg} \cdot \text{ml}^{-1}$ of sodium borohydride was treated with 2–33% of 1,4-butanediol diglycidyl ether at room temperature for 16 hours leading to formation of oxirane-alginate. The reaction mixture was adjusted to pH 9.0, dialyzed overnight against deionized water and

subsequently tested for its gel forming capacity. Four volumes of oxirane-alginate prepared at 2% bisepoxide was adjusted to a pH value in the range 4.5–9.0 and mixed with one volume of crude glucoamylase, three-fold diluted and pH-adjusted. The coupling took place overnight at 4°C and after dialysis against 0.05 M-sodium acetate, pH 4.5, the mixture was used without further treatment for formation of calcium alginate-glucoamylase gels.

3.3.4. *Coupling to cyanogen bromide activated alginate*

Activation of alginate was performed in principle as described by MARCH et al. for Sepharose (15). A 3% solution of sodium alginate, dialyzed against 1 M-sodium carbonate, was treated with $8\text{--}80 \text{ mg} \cdot \text{ml}^{-1}$ of cyanogen bromide. After two minutes reaction the mixture was dialyzed against 0.1 M-sodium bicarbonate, pH 8.0, in the cold for two hours. Crude glucoamylase, which had been dialyzed against the same buffer, was added to 13 volumes of the activated alginate. The coupling mixture was left overnight at 4°C with stirring and finally subjected to gel formation.

3.3.5. *Coupling to alginate by means of carbodiimide*

A 1:10 (v/v) mixture of glucoamylase and 3% sodium alginate was adjusted to pH 4.75 and four portions of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide ($9 \text{ mg} \cdot \text{ml}^{-1}$) were added at intervals of 20 minutes. The pH was maintained at 4.75 during 100 minutes by titration with 0.1 N HCl and gel formation was carried out immediately afterwards.

3.3.6. *Coupling to azido-alginate*

The preparation of azido-alginate was accomplished by a procedure modified from the synthesis of azido-agarose by PARIKH et al. (20). A 2.6% solution of sodium alginate was made 0.02 M in sodium periodate and left for two hours at room temperature. A 0.1 M-solution of adipic dihydrazide, pH 5.0, was added to give a final concentration of 0.045 M and the reaction took place for two hours at room temperature

with stirring. The subsequent steps of reduction to hydrazido-alginate and its conversion to azido-alginate, were carried out as described earlier (20). The resulting solution of azido-alginate was diluted 10 fold with 0.2 M-sodium bicarbonate, pH 8.0 or 9.0, and 70 μ l of crude glucoamylase, three fold diluted and adjusted to the same pH, was added per milliliter of azido-alginate. The mixture were left overnight at 4 °C, dialyzed against 0.01 M-sodium acetate, pH 4.5, and lyophilized.

3.4. Coupling capacity

The amount of reactive groups in the different support materials was estimated by coupling with a low molecular weight compound, glycyl-L-leucine amide. The conditions were identical with those used for reaction with proteins and glycyl-L-leucine amide were present in a 5–16 fold molar excess with respect to sugar units. Unreacted amino compound was removed by dialysis and the amount of polymer-bound dipeptide amide was measured by amino acid analysis (27).

3.5. Analyses

For routine determinations of the enzymic activity 0.015 M-maltose in 0.1 M-sodium acetate, pH 4.3, 0.1 M-calcium chloride, was used as substrate. One unit was defined as the amount of enzyme, which hydrolyses 1 μ mole of maltose per minute at 25 °C. The enzymic reaction was inhibited after 30 minutes incubation by addition of 1.5 volumes of 1.66 M-Tris, pH 7.6. The amount of glucose released was subsequently determined using the reagents of the Merck System Glucose kit made up in 0.1 M-Hepes, pH 7.6. When immobilized glucoamylase was assayed, the enzyme-containing particles were weighed out and incubated with substrate during gentle horizontal shaking.

The oligodextrin hydrolysis was determined using 0.7% partially hydrolyzed starch as substrate in an assay performed as the assay with maltose. One oligodextrin hydrolysis unit was defined as the amount of enzyme releasing 1 μ mole of glucose per minute at 25 °C and at pH 4.3. A mixture of oligodextrins was prepared by adding 135 μ l of Termamyl 60 L to one litre of a

10% suspension of starch and heating at 120 °C. After incubation for 10 min another 135 μ l aliquot of Termamyl was added, the mixture rapidly cooled, and centrifuged. The supernatant was incubated for 15 minutes at 120 °C and after another centrifugation the resulting supernatant was used as the stock solution of oligodextrin substrate. A typical preparation was subjected to gel filtration (6) and the contents of compounds of different degrees of polymerization were found to be: DP 1 = 6%, DP 2 = 18%, DP 3 = 20%, DP 4 = 8.6%, DP 5 = 27%, DP 6–11 = 10%, higher DP = 10.4%.

The protein content of gel particles was determined after lyophilization by amino acid analysis (27), norleucine being added as internal standard.

The content of dextran in dextran-glucoamylase conjugates was determined by the phenol-sulphuric acid procedure using glucose as standard (5).

4. RESULTS

4.1. Polymerization and entrapment of glucoamylase

Intermolecular cross-linking of glucoamylase by 1% or 2% of glutaraldehyde in the pH range 6.0–9.0 was not accompanied by formation of insoluble material. At pH 6.0 the polymeric products retained 55% and 25% of the initial activity using 1% and 2% glutaraldehyde, respectively, whereas at higher pH values a further decrease in activity occurred (Figure 1). Entrapment in alginate gel particles decreased the activity 7–15 fold with the highest residual activity resulting from glucoamylase previously cross-linked at pH 7.0 (Figure 1). The activity of the polymerized glucoamylase decreased with increasing concentration of glutaraldehyde in the range 0.02–2%, while calcium alginate particles containing glucoamylase showed the highest residual activity when the enzyme was previously treated with 1% of glutaraldehyde (Figure 2). These findings emphasize that investigations of the entrapped derivatives are needed to select optimal cross-linking conditions leading to enzyme derivatives sufficiently large and still active. The residual activity of these gel entrapped forms corresponded to only 1%–4% of the

Figure 1. The percentage of the initial activity toward maltose remaining after the cross-linking of crude glucoamylase by glutaraldehyde 1% (□) and 2% (■) (left ordinate).

The apparent activity displayed by derivatives cross-linked at 1% (○) and 2% (●) of glutaraldehyde after incorporation in the gel particles is given in $U \cdot g^{-1}$ of hydrated particles (right ordinate). Furthermore, from these values were calculated recovery of activity for entrapped derivatives in per cent of the activity initially present prior to cross-linking (outer right ordinate).

Figure 2. The percentage of the initial activity toward maltose retained in crude glucoamylase cross-linked at pH 6.0 and at 0.02–2% of glutaraldehyde (□) (left ordinate).

The apparent activity displayed by the cross-linked derivatives after gel entrapment is given in $U \cdot g^{-1}$ of hydrated particles (○) (right ordinate). Furthermore, from these values were calculated the recovery of activity for entrapped derivatives in per cent of the activity initially present prior to cross-linking (outer right ordinate).

activity of unmodified glucoamylase (Figures 1 and 2).

Glucoamylase was also polymerized by treatment with 0.04–0.18 M-dimethylsuberimide. At higher concentrations, the reaction mixture became turbid, but no extensive precipitation occurred and at the end of the reaction period (2½ hours) 80–90% of the activity was still present. However, the gel entrapped derivatives displayed only 0.5–1.5% of the original activity. Entrapment of glucoamylase polymerized by dimethylsuberimide at concentrations higher than 0.03 M resulted in retention of 0.5% of the activity corresponding to $0.02 U \cdot g^{-1}$ of gel particles.

The ability of calcium alginate to retain cross-linked glucoamylase was tested by measuring enzymic activity in the supernatant of an agitated suspension of gel particles. In the case of dimethylsuberimide cross-linked glycoamylase, no active enzyme was detected in the solution surrounding the gel particles. However, glutaraldehyde cross-linked preparations were released through the gel micropores in small amounts, corresponding to less than 2% of the entrapped activity.

4.2. Dextran coupling and entrapment of glucoamylase

In preliminary experiments dextran activated with cyanogen bromide at a molar ratio of CNBr:sugar units of 1:1 and 1:7 contained 13% and 1.3%, respectively, of activated monosaccharide residues as determined by reaction with glycyl-L-leucine amide. The dextran of 13% coupling capacity was chosen for further studies. The reaction of dextran with glucoamylase resulted in a mixture of water-soluble and water-insoluble dextran-enzyme conjugates with a recovery of activity varying from 90–100% when crude dialyzed enzyme or purified enzyme was used in the coupling step and from 65–82% when the crude glucoamylase was used (Table I). The activity of dextran-coupled enzyme preparations decreased by 15%, when the pH of the coupling step was increased from pH 7.0 to 8.0 and by 20% when the pH was increased from 7.0 to 9.0. The content of dextran in the conjugates was in the range 46–82% (Table I).

Upon entrapment, highest activity of the

Table I.

Preparation and entrapment of dextran-glucoamylase.

Enzyme preparation	Enzyme in coupling mixture (U · g ⁻¹ of dextran)	Dextran-glucoamylase		Calcium alginate dextran-glucoamylase	
		Recovery of activity (%)	Dextran content (wt. pct.)	Activity of gel particles (U · g ⁻¹)	Recovery of activity (%)
crude	380	82	82	0.38	8.4
crude	900	70	63	0.45	4.2
crude	1800	65	46	0.28	1.5
crude, dialyzed	220	100	72	0.48	18.2
crude, dialyzed	480	98	64	0.56	10
purified	180	92	64	0.55	26

The commercial enzyme, Amyloglucosidase AMG 200 L, contains 220 U · ml⁻¹ towards maltose. The crude enzyme is the commercial enzyme which has been three fold diluted and adjusted to pH 7.0 prior to dextran-coupling. Occasionally this enzyme was dialyzed against 0.01 M-sodium phosphate, pH 7.0, prior to coupling. The purified glucoamylase is the G I component (14, 28) isolated in homogeneous form from Amyloglucosidase 200 L. Dextran-coupling was performed at pH 7.0. For entrapment lyophilized dextran-glucoamylase was mixed at a ratio of 0.8–1.3 for enzyme derivative to sodium alginate (wt/wt). The dextran content was determined for an aqueous suspension of enzyme derivative using the phenol-sulphuric acid procedure and expressed in weight per cent of the protein plus carbohydrate contents. The activity toward maltose recovered after dextran-coupling was determined on an aliquot of an aqueous suspension of the enzyme derivative. The activity toward maltose after entrapment was expressed in units per gram of hydrated gel particles. All recoveries are given in percentage of the activity used for dextran-coupling and were in the case of calcium alginate-glucoamylase preparations calculated from the amount of gel particles formed.

resulting enzyme-containing gels was attained with dextran-coupled crude dialyzed glucoamylase or purified glucoamylase. Using crude glucoamylase the greatest activity was retained when the enzyme derivative of intermediate dextran content was used (Table I). The lower yields obtained when higher amounts of crude enzyme were used for dextran coupling may arise from competition between glucoamylase molecules and other components present in the crude enzyme preparation for reactive groups in the dextran.

The apparent activity of the enzyme containing gels depended on the amount of enzyme derivative added to the alginate solution to be precipitated. Thus increasing the amount of dextran-glucoamylase in the range 0.2–1.8 g · g⁻¹ of sodium alginate resulted in increasing activities from 0.2–0.8 U · g⁻¹ of gel with corresponding recoveries of activity decreasing from 30 % to 18 % (Figure 3). The oligodextrin hydrolysis, by the same entrapped derivatives, also showed an increase in activity with increasing amounts of enzyme derivative used for entrapment (Figure 3).

Figure 3. The recovery of activity for gel incorporated dextran-coupled crude glucoamylase (dextran content: 63 %, Table I) given in per cent of the activity toward maltose (□) or partially hydrolyzed starch (■) used for the entrapment at different ratios of dextran-glucoamylase : alginate (wt/wt) (left ordinate). The apparent activity for maltose (○) and partially hydrolyzed starch (●) is also shown in U · g⁻¹ of hydrated particles (right ordinate).

Figure 4. Entrapment of crude glucoamylase (11 U), dextran-coupled crude glucoamylase (dextran content: 63 %, Table I) (8 U) and dextran-coupled purified glucoamylase (Table I) (8 U) using 10 ml of 3 % sodium alginate.

The amount of non-entrapped enzyme was determined in supernatant number 1 using maltose as substrate. The particles were subsequently washed in seven steps each consisting in stirring for 1 hour in 50 ml of 0.1 M-sodium acetate pH 4.3, 0.1 M-calcium chloride. The amount of activity liberated for crude glucoamylase ($\blacktriangle$), dextran-coupled crude glucoamylase ($\blacklozenge$) and dextran-coupled purified glucoamylase ($\odot$) were added with the amount found for previous supernatants and expressed in per cent of the amount of activity subjected to entrapment. The apparent activity of the resulting particles was $0.006 \text{ U} \cdot \text{g}^{-1}$ for crude glucoamylase, $0.16 \text{ U} \cdot \text{g}^{-1}$ for dextran-coupled crude glucoamylase, and $0.36 \text{ U} \cdot \text{g}^{-1}$ for dextran-coupled purified glucoamylase, which corresponds to recoveries of 0.4 %, 19 % and 37 % of the starting activity, respectively.

The retention of various glucoamylase preparations in calcium alginate gels was studied by entrapping unmodified crude glucoamylase (molecular weight less than 100,000 (28)), dextran-coupled crude glucoamylase and dextran-coupled purified glucoamylase under identical conditions and following the enzymic activity released in the washing steps. Essentially, all of

the unmodified enzyme was easily washed out of the gel, whereas only about half of the activity was liberated in the case of the two dextran-bound enzyme preparations (Figure 4). The washed gel particles containing dextran-coupled purified glucoamylase showed an activity corresponding to 37 % and particles containing dextran-coupled crude enzyme an activity corresponding to 18 % of the amounts of respective derivatives used for the entrapment. Prior to entrapment the specific activities of the dextran-bound purified and crude enzyme were 100 % and 80 %, respectively, of the values determined for the unmodified enzyme preparations, while upon entrapment the specific activities based on protein contents decreased to 70 % of the purified and 34 % of the crude enzyme. This suggests that the derivative of crude glucoamylase is less accessible to the substrate than the derivative of the purified enzyme.

After dextran-coupling of crude glucoamylase the specific activity toward maltose or oligodextrins varied in general from 65 % to 100 % of the initial value. Upon entrapment, however, specific activities would be 30–60 % with maltose and 5–20 % with oligodextrins of the values for unmodified glucoamylase. Again for entrapped dextran-bound purified glucoamylase the corresponding specific activities were slightly higher, 65 % and 23 %, respectively. Finally it must be mentioned that changes in the gelling properties of alginate occasionally resulted in variations in residual activities obtained with the same dextran-glucoamylase preparation.

4.3. Coupling of glucoamylase to aminoethylcellulose

In an attempt to prepare derivatives of glucoamylase retained to an even higher extent in the gels the enzyme was bound to insoluble, glutaraldehyde-activated aminoethylcellulose with a maximal activity of $0.6 \text{ U} \cdot \text{g}^{-1}$ of resin (Figure 5). Such derivatives could be permanently entrapped by the calcium alginate procedure, and they have also been used for coentrapment with yeast (manuscript in preparation). It is a limitation, however, that the activity of the aminoethylcellulose-glucoamylase is rather low and consequently large amounts of conjugate are required to prepare alginate gels of reasonably

Figure 5. The recovery of the activity measured with maltose as substrate in aminoethylcellulose-glucoamylase prepared at different ratios of enzyme to resin (■) (left ordinate).

The activity of the coupled product is given in $\text{U} \cdot \text{g}^{-1}$ of resin (●) (right ordinate).

The ratio of activities (U/U) for partially hydrolyzed starch and maltose is calculated from activities measured at the conditions given in section 3.5 (○) (left ordinate).

high activity. Thus 2 g of aminoethylcellulose-glucoamylase and 10 ml of 3 % sodium alginate gave particles of $0.05 \text{ U} \cdot \text{g}^{-1}$. Furthermore, while the ratio of activities for partially hydrolyzed starch and maltose of the resin-bound glucoamylase was about 4 as compared to 7 for the unmodified enzyme (Figure 5), this value decreased to about 0.7 upon entrapment.

4.4. Coupling of glucoamylase to alginate

In a different approach enzyme containing gels were prepared from alginate to which

glucoamylase had been covalently bound prior to the precipitation by calcium ions. In order to preserve the gel forming capacity of the alginate, however, the reactive intermediates azido-alginate, oxirane-alginate and cyanogen bromide treated alginate, had to be synthesized under very gentle conditions in comparison to the procedures normally employed for activation of polysaccharides. These mild procedures are probably reflected in the low coupling capacities of the alginate derivatives (Table II).

Conversion of 2 % sodium alginate to oxirane-alginate by 2–33 % 1,4-butanediol diglycidyl ether made the alginate solution increasingly more viscous as compared to an untreated control. The coupling capacity obtained at 2 % of the bisepoxide was acceptable and at the same time the increase in viscosity was rather low, easily allowing precipitation as spherical particles. Coupling reactions with oxirane-polysaccharides are normally carried out at pH 8.5–10.0 (23). However, in the case of sodium alginate it appeared that glycyl-L-leucine amide was incorporated in higher yields at pH 7 and the oxirane-alginate-glucoamylase leading to the highest enzymic activity of gel particles was also prepared at this pH (Figure 6). The residual activity in oxirane-alginate-glucoamylase gels was 0.2–1.3 % (Figure 6).

The conventional cyanogen bromide activation of polysaccharides could also be performed with alginate. When 2 % sodium alginate was activated the concentration of cyanogen bromide had to be kept below $30 \text{ mg} \cdot \text{ml}^{-1}$ in order to preserve the gel forming properties of the polymer. While 80 % of the activity was retained after coupling of glucoamylase to the

Table II.

Coupling of glucoamylase and glycyl-L-leucine amide to alginate.

Type of derivative	Activity ($\text{U} \cdot \text{g}^{-1}$)	Coupling capacity (%)
Oxirane-alginate	0.18	17
CNBr-activated alginate	0.14	0.5
Carbodiimide-activated alginate	0.14	1.2
Azido-alginate	0.19	2.5

The activity after entrapment of the various alginate-glucoamylase preparations was expressed as units per gram of hydrated gel particles using maltose as substrate. The coupling capacity is given as the percentage of alginate sugar acid units reacting with glycyl-L-leucine amide.

Figure 6. The recovery of activity after oxirane-alginate-coupling of glucoamylase in the pH range 4.5–9 (■) is given in per cent of the activity initially present (left ordinate).

The apparent activity of the enzyme containing particles for maltose in $\text{U} \cdot \text{g}^{-1}$ of hydrated particles (●) (right ordinate) and the recovery of activity as compared to the amount of unmodified glucoamylase used for alginate coupling is indicated (outer right ordinate). The coupling capacity of oxirane-alginate for glycyl-L-leucine amide is given as the number of glycyl-L-leucine amide molecules reacting per 100 sugar acid units (×) (left ordinate).

activated alginate, the alginate-glucoamylase gels showed an activity of only 1 % of the starting activity.

Alginate-glucoamylase prepared by carbodiimide-coupling readily formed gels. This suggested the degree of modification of the carboxyl groups of the alginate to be quite low, and this was confirmed by the low coupling capacity for glycyl-L-leucine amide (Table II). The gel particles showed a residual enzymatic activity of 1.3 % of the starting activity.

For preparation of azido-alginate the polymer was initially oxidized by various concentrations of sodium periodate in the range 0.0035–0.13 M. Subsequent precipitation by calcium ions, however, was only successful when periodate concentrations below 0.02 M had been used. After conversion to azido-alginate coupling of enzyme at pH 8.0 or 9.0 resulted in a recovery of about 30 % of the activity in the case of crude glucoamylase, while the purified glucoamylase was coupled without any significant loss of activity. In spite of the capability of the alginate to form gels after oxidation, the alginate-glucoamylase derivatives would only precipitate

with great difficulty. Spherical particles were easily obtained, however, from a mixture of 0.8 % alginate-glucoamylase and 2.2 % unmodified alginate.

The different alginate-glucoamylase conjugates showed activities about 0.1–0.2 $\text{U} \cdot \text{g}^{-1}$ of particles (Table II) corresponding to recovery of initial activity from 0.06 % to 3 %. The oxirane-alginate-glucoamylase and the azido-alginate-glucoamylase did not release detectable amounts of enzyme, whereas on a few occasions up to 10 % of the activity displayed by the gel particles made from cyanogen bromide activated alginate-glucoamylase and carbodiimide alginate-glucoamylase was liberated in the assay mixture.

4.5. Continuous hydrolysis of oligodextrins

Spherical particles of calcium alginate containing entrapped dextran-glucoamylase were packed in a small column ($0.9 \times 15 \text{ cm}$) and used as an enzyme reactor. At pH 4.3 and at room temperature partially hydrolyzed starch, 1.4 % (wt/v), could be essentially converted to glucose at a substrate residence time of 15 hours. The activity of the enzyme reactor did not decrease during two months of continuous operation. Reactors packed from carbodiimide-coupled alginate-glucoamylase or entrapped dimethylsuberimidate cross-linked glucoamylase have been working for 1 month with no loss in enzyme activity.

5. DISCUSSION

The enzymic activity of a crude preparation of glucoamylase was well retained after the various types of chemical modifications which were attempted to increase the size of the enzyme molecule (Table III). Modification of the purified enzyme showed an increase in comparison to modification of the crude enzyme in recovered activity from 70 % to 98 % by dextran-coupling and from 30 % to 100 % by azido-alginate-coupling. The residual activity and the specific activity after entrapment were also higher in the case of the purified enzyme. These improvements may reflect that the more stable of the two major *A. niger* glucoamylase components, G I, (14, 28) was used as the purified enzyme, whereas the crude enzyme contained essentially

Table III.

Calcium alginate entrapped derivatives of crude glucoamylase.

Type of derivative	Activity recovered after chemical modification (%)	Activity recovered after entrapment (%)	Activity in gel particles (U · g ⁻¹)
Glutaraldehyde-polymerized	40	4.2	0.65
Dimethylsuberimide-polymerized	90	1.5	0.02
Dextran-coupled	70	18	0.82
Aminoethylcellulose-coupled	6	0.6	0.05
Oxirane-alginate-coupled	80	1.3	0.18
CNBr-alginate-coupled	80	1.0	0.14
Carbodiimide-alginate-coupled	—	1.3	0.14
Azido-alginate-coupled	30	3.0	0.19

For each type of derivative the one resulting in calcium alginate gels of highest apparent activity has been included in the list. The recovery of activity in gel particles, however, was normally higher than the indicated values when less glucoamylase derivative was subjected to entrapment. The recovery of activity after chemical modification and after entrapment of the resulting derivatives is given in percentage of the activity originally used for the chemical modification. Maltose was the substrate in all cases and after entrapment the activity is expressed in units per gram of hydrated gel particles.

equal amounts of the G I, and the less stable G II component. However, the derivatives obtained with the crude enzyme still yielded satisfactory activities. The entrapment of various glucoamylase derivatives caused apparent reductions in the activities due to diffusional limitations and steric hindrance of the enzyme substrate interactions. Thus the activity of the unmodified enzyme was about 7 fold as high for oligodextrins as for maltose, whereas gels containing dextran-glucoamylase or aminoethylcellulose-glucoamylase gave ratios of oligodextrin hydrolysis units to maltose hydrolysis units varying from 0.7 to 1.4. Attachments of subtilisin to soluble oligosaccharides or polysaccharides have previously been demonstrated to have similarly minor effects on the activity, while recoveries for corresponding insoluble derivatives were low due to the microenvironmental effects mentioned above (25, 26). In the present case the decrease in activity of glucoamylase derivatives due to these effects will probably not exclude their practical utility. It is clear from the compilation in Table III that, after entrapment, dextran-bound and glutaraldehyde-polymerized glucoamylase were most active. The dextran-glucoamylase was the derivative preferred for studies on coimmobilization with yeast since its entrapment occurred in high yields and the conjugates were fully

retained, in comparison to glutaraldehyde cross-linked glucoamylase which leaked slowly from the gel matrix. Thus dextran-glucoamylase most satisfactorily combined retention of enzymic activity with formation of sufficiently large conjugates. Gel beads containing this derivative enabled continuous hydrolysis of oligodextrins to glucose, at conditions comparable to hydrolysis of beer wort dextrins in brewing. The utility of dextran-glucoamylase after coimmobilization with brewers yeast for production of low calorie beer has also been demonstrated (manuscript in preparation).

The information gained on how to prepare chemical derivatives of glucoamylase which are retainable in gels might be useful in other processes involving the combined action of microorganisms and enzymes. For example penicillin G has been produced by entrapped *Penicillium chrysogenum* (24) while the hydrolysis of the natural penicillin to 6-aminopenicillanic acid by immobilized penicillin acylase has been independently reported (13, 31). A coimmobilisate of the *Penicillium chrysogenum* and the penicillin acylase may thus be suggested for production of 6-aminopenicillanic acid, a precursor of semisynthetic penicillin. Furthermore, as it is frequently advantageous to coimmobilize two or more enzymes (19), it is likely that

similar advantages may be gained by coimmobilizations of living microorganisms with enzymes in the cases of enzyme catalyzed reactions followed by microbial conversion of the products or vice versa.

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