PREPARATION OF CARBOXYMETHYL-CELLULOSE GELS AND THEIR USE FOR IMMOBILIZATION OF AMYLOGLUCOSIDASE (E.C.3.2.1.3)

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A carboxymethyl-cellulose gel of high hydration degree to be used for immobilization of enzymes was prepared by precipitation from the solution of a sodium salt of highly substituted carboxymethyl-cellulose, treatment of the precipitated gel with formaldehyde, and freezing. The gel was converted into its azide and used for the immobilization of amyloglucosidase (E.C.3.2.1.3). The determination of the activity ratio showed that, compared to the agarose gel, there is almost no sterical hindrance caused by the carboxymethyl-cellulose support. The latter equals agarose gels in this property yet it shows better mechanical characteristics. The properties were compared of soluble amyloglucosidase with the properties of amyloglucosidase bound to the carboxymethyl-cellulose gel, DEAE-cellulose, CM-cellulose, and agarose. The K_m-values of the immobilized enzyme were lower than the K_m-values of the soluble enzyme in all cases. The pH-optimum of the carboxymethyl-cellulose gel-bound enzyme and of the CM-cellulose-bound enzyme was shifted by 1·1 and 0·6 units, respectively toward the alkaline range. The thermal stability of the enzyme was not affected by its immobilization.

Carboxymethyl-cellulose is one of the supports commonly used for the immobilization of enzymes. It possesses almost all the favorable properties of agarose gels and shows moreover a higher mechanical endurance. The only disadvantage of carboxymethyl-cellulose compared to agarose is its highly compact structure which unfavorably affects the course of the enzymatic reaction. Carboxymethyl-cellulose has been therefore replaced by other materials (polyacrylamide, nylon, *etc.*) showing similar mechanical characteristics and a less compact structure.

Mitz and Summaria[1] have shown that the specific activity of enzymes bound to carboxymethyl-cellulose strongly depends on the degree of hydration of the support. The hydration degree depends on the porosity of the material and its hydrophilic character. The hydration of carboxymethyl-cellulose can be increased by its substitution; simultaneously the support becomes also more hydrophilic. The chief disadvantage involved in the increasing of the hydration degree of carboxymethyl-cellulose is that the derivatives of a higher substitution degree are soluble in alkaline media and form in acid media a sticky, viscous gel, entirely unsuitable for enzyme reactors of all types.

This paper describes the preparation of a highly substituted (substitution degree 0·7) carboxymethyl-cellulose gel, containing 90–92% of water and showing good mechanical properties from the viewpoint of use in enzyme reactors of the column type or the stirred type. We showed that the steric effect of the support on the course

of the enzymatic reaction is as small as that of agarose gels commonly used; the gel retains all the good mechanical properties characteristic of cellulose derivatives at the same time.

Amyloglucosidase (E.C.3.2.1.3), one of the technically important enzymes, whose immobilization has been devoted considerable attention during the past few years[2-4] was chosen by us as a model for a comparison of immobilization of enzymes on the carboxymethyl-cellulose gel described with the immobilization on other supports commonly used (agarose, carboxymethyl-cellulose Whatman, DEAE-cellulose). We compared the stability, pH-dependence of enzymatic activity, and K_m-value of amyloglucosidase immobilized on various supports with the same properties of soluble amylglucosidase.

EXPERIMENTAL

Preparation of Carboxymethyl-cellulose Gel

a) Precipitation of soluble carboxymethyl-cellulose: the sodium salt (10 g) of carboxymethyl-cellulose (Lovosa, substitution degree 0·65 ± 0·05) was dissolved in 100 ml of water and the viscous solution was poured into 100 ml of 10% sulfuric acid at room temperature (the rate of the pouring and stirring determines the size of the carboxymethyl-cellulose gel fibers). After the total quantity of carboxymethyl-cellulose had been added, the mixture was stirred for one more hour and the gel was centrifuged off afterwards. The gel was suspended in 200 ml of water and centrifuged off again. This washing procedure was repeated until the acidic reaction of the supernatant disappeared.

b) Preparation of "spongy" carboxymethyl-cellulose gel: the washed carboxymethyl-cellulose gel was frozen at −22°C (12 h) and then allowed to thaw at room temperature. This procedure was repeated once more. The gel was filtered off and homogenized to the desired particle size in a laboratory blender. The fines were removed from the gel by settling.

c) Treatment of gel with formaldehyde: As will be described below (see "Discussion"), the properties of the gel are markedly improved if the gel precipitated in acidic medium is treated with formaldehyde before being frozen. The entire quantity of the precipitated gel was mixed with 200 ml of 1M-HCl containing 200 ml of 32% formaldehyde. The mixture was stirred 2 h at room temperature, the gel was filtered off, and mixed with a large volume of water.

The dry weight of the gel thus prepared is 1·58% and the gel contains 2·09 mmol of carboxyl groups per 1 g of dry weight (gel not treated with formaldehyde). The dry weight of the formaldehyde-treated gel is 8·22% and the carboxyl group content is 2·06 mmol per 1 g of dry weight.

Preparation of Gel for Binding the Enzyme

Carboxymethyl-cellulose chloride was prepared by the method of Arsenis and McCormick[5]. The chlorine content of the acyl chloride was 1·86 mmol per 1 g of dry weight (formaldehyde-treated gel). The hydrazide was prepared from the acyl chloride by the modified method used for the preparation of carboxymethyl-cellulose amides[5]. The determination of nitrogen content shows that the derivative contains 1·9 mmol of hydrazine per 1 g of dry weight (formaldehyde-treated gel).

Preparation of Carboxymethyl-cellulose Ester

The dry formaldehyde-treated gel (10 g, dried *in vacuo* over P_2O_5 at 60°C) was refluxed 18 h with 200 ml of dry ethanol containing 1 ml of concentrated sulfuric acid. The esterified gel was filtered off, washed three-times with 200 ml of ethanol and twice with 200 ml of water. The concentration of ester groups was not determined. After conversion into its hydrazide the gel contained 1·62 mmol of hydrazine per 1 g of dry weight. The preparation of the hydrazide from the ester and of the azide from the hydrazide was effected by a common method[1], as well as the binding of the enzyme to the azide.

Determination of Amyloglucosidase Activity

The enzyme solution (2 ml) or the same volume of a suspension of the immobilized enzyme was incubated with 2 ml of 2% solution of soluble starch, 1 h at 30°C. The reaction was discontinued by heating (10 min) if the soluble enzyme was tested, or by centrifugation (15000 *g*, 3 min) in experiments with the immobilized enzyme. Glucose resulting from the enzymatic reaction was determined in the filtrate (supernatant) colorimetrically using the system glucose oxidase–peroxidase–*o*-dianisidine. The concentration of the protein was calculated from the nitrogen content and the effectiveness of the binding was expressed by the activity ratio[6] ϱ (%) = (specific activity of bound/specific activity of native enzyme) × 100.

The determination of the thermal stability was effected by incubation of the enzyme suspension at 50, 60, and 70°C and determination of residual activity of the enzyme at regular time intervals (30 min). The determination of K_m-values was carried out in the same manner as the determination of the enzyme activity; substrate solutions of different dilution were used. In view of the character of the substrate the K_m-value was expressed in per cent. The calculation was carried out by the method of least squares from linear plots of $1/v$ *versus* $1/S$, of v *versus* v/S, and of S/v *versus* S.

The dependence of enzyme activity on pH was measured in the range pH 2·2—9·5 (0·1M acetate or phosphate with respect to the desired pH-value). The determination was carried out in the same manner as the determination of the enzyme activity. The determination of the dependence of enzyme activity on pH could not be carried out with the enzyme immobilized by adsorption to DEAE-cellulose since the adsorption of the enzyme is strongly pH-dependent; hence, the elution of the enzyme would be different at different pH-values and the results would be erroneous.

RESULTS AND DISCUSSION

The hydrophilic, spongy carboxymethyl-cellulose gel, prepared by treatment of the soluble sodium salt of highly substituted carboxymethyl-cellulose (Lovosa) in an acid medium with subsequent freezing, contains a large quantity of water in hydrated state. This quantity of water can reach the value of even 98% and corresponds to the characteristics of the thinnest agarose gels. We examined the filtrating properties of this gel at different hydrostatic pressures and evaluated the results by using the known relation $U = Kdp/L$, where U is the linear flow rate (cm/min), dp the hydrostatic pressure (cm of H_2O column), L the height of the gel column, and K a constant characterizing the gel. Constant K does not depend on the hydrostatic pressure if the gel is incompressible (such as, *e.g.* Sephadex G-25, cellulose derivatives,

etc.) If the gel is compressible, constant K is dependent on the pressure and starts decreasing at a certain value of the pressure; this is paralleled by a sharp drop of the flow rate. As regards the carboxymethyl-cellulose gel, constant K is independent of the pressure up to a value of 18 cm of water column (Fig. 1), *i.e.* the gel cannot be used at a higher hydrostatic pressure. Hence, even in this property the gel equals the thinnest agarose gels. We looked therefore for a procedure which would improve his flow characteristics. We had the best experience with formaldehyde treatment in an acid medium immediately after the precipitation of the gel before the thawing. The formaldehyde-treated gel is slightly less hydrated than the untreated gel, it contains approximately 8% of dry weight, yet his flow properties are far better. Constant K is independent of hydrostatic pressure up to a pressure of 100 cm of water column; this is suitable for all types of enzyme reactors. Hydration is only a little lower than with the most common agarose gel, Sepharose 4 B. A comparison of both types of carboxymethyl-cellulose gels is given in Table I. In view of the favorable properties of the formaldehyde-treated gel, we used exclusively this type in all our subsequent experiments.

To verify the possibility of immobilization of enzymes on the carboxymethyl-cellulose gel, we converted the free carboxyl groups both to the acyl chloride and to the

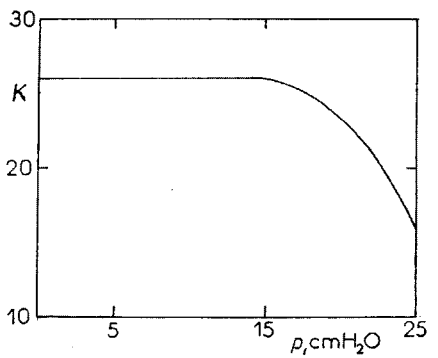

FIG. 1

Dependence of Flow-rate Constant K on Hydrostatic Pressure for Unstabilized Carboxymethyl-cellulose Gel

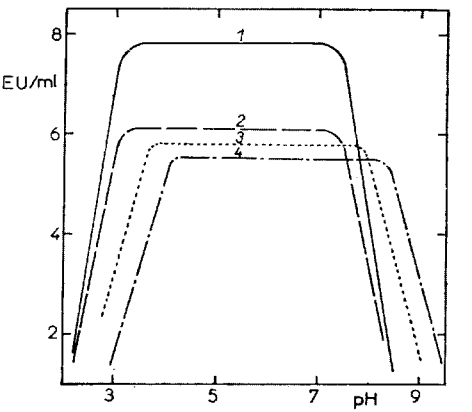

FIG. 2

Dependence on pH of Enzymatic Activity of Amyloglucosidase (AMG) and Amyloglucosidase Immobilized on Different Supports

1 Soluble AMG, *2* AMG-agarose, *3* AMG-CMC-gel, *4* AMG-CM-70.

ester. We prepared hydrazides and azides from both derivatives by the known procedure[1,5]. The possibilities of rapid hydrolysis[5] of the acyl chloride must be respected, however, and anhydrous hydrazine must be used; this slightly complicates the use of this method. The hydrazides were then converted into azides by the known method; the latter were employed for the immobilization of amyloglucosidase (E.C./3.2.1.3). Except for the ester groups, the concentration of substituents was determined at each step. To compare the effectiveness of binding of amyloglucosidase to the carboxymethyl-cellulose gel with the remaining methods described, we coupled the same enzyme to cyanogen bromide[7] activated agarose, and to DEAE-cellulose[8]. In all cases were determined the quantity of the protein bound, the activity of the

TABLE I

Comparison of Formaldehyde-treated Carboxymethyl-cellulose Gel with Untreated Gel

Gel	Untreated	Formaldehyde--treated
COOH-content	2·09 mmol/g	2·06 mmol/g
Dry weight	1·58%	8·74%
Usable up to a hydrostatic pressure of	18 cm of H_2O	more than 100 cm of H_2O column

TABLE II

Immobilization of Amyloglucosidase (E.C.3.2.1.3) on Different Supports

Support	Yield of activity %	Protein bound mg/g	Activity ratio %
CMC-gel[a]	47·5	30	82·32
CMC-gel[b]	40·6	27·6	76·27
CMC-CM-70	23·1	26·2	45·93
Sepharose 4B	45·8	27·2	83·32
DEAE-cellulose	53·9	32·5	86·42

[a] The carboxymethyl-cellulose gel (CMC-gel) was converted into its azide *via* the acyl chloride as intermediary product; [b] The carboxymethyl-cellulose gel was converted into its azide *via* its ester as intermediary product.

immobilized enzyme, and the yield of the binding with respect to the activity of the soluble enzyme subjected to immobilization. The activity ratio, which can serve as a criterion of sterical hindrances caused by the support, was also calculated. The results are given in Table II.

The results of the immobilization experiments show that the use of the carboxymethyl-cellulose gel, whose preparation was described in this paper, does not bring about a sterical inhibition of the enzyme by the support, at least (according to the data given in Table II) to an extent exceeding that observed with agarose. Compared to carboxymethyl-cellulose for chromatography (Whatman CM 70), a significant improvement of the activity ratio was obtained after the hydration of the support had been increased. We also showed that the carboxymethyl-cellulose gel practically equals in hydration and porosity the agarose gels; this is indicated by the same activity

TABLE III

K_m-Value of Amyloglucosidase (AMG) Immobilized on Different Supports And of Soluble Amyloglucosidase

Type of enzyme	K_m	K_m/K_m Sol.AMG
Soluble AMG	0·454	1
AMG-agarose	0·013	0·029
AMG-DEAE-cellulose	0·096	0·211
AMG-CM-70	0·189	0·416
AMG-CMC-gel	0·189	0·416

TABLE IV

Stability of Amyloglucosidase (AMG) Bound to Different Supports

The results are given in % of input activity. Incubation 30 min.

Temperature, °C	Relative enzymatic activity				
	sol.AMG	AMG-agarose	AMG-DEAE-C	AMG-CM-70	AMG-CMC-gel
50	100	100	100	100	100
60	100	100	100	100	100
70	55·9	62·4	57·1	55·2	58·9

ratio obtained with both types of gels; the carboxymethyl-cellulose gel, however, is superior to the highly hydrated agarose gels in its mechanical properties which enable higher hydrostatic pressures to be applied to the columns. This paves the way to the use of this gel in industrial operations.

A great number of data have been recorded showing that the binding of the enzyme to the support considerably affects its K_m-value (*e.g.*[9]); it is therefore worthwhile to compare the K_m-values of enzymes immobilized on various supports. We have shown in our experiments (Table III) that the K_m-value of amyloglucosidase immobilized on all supports used is lower than the K_m-value of the native, soluble enzyme. The greatest difference in K_m-values was observed to exist between the native enzyme and the enzyme bound to agarose: this difference is of one order. Interest deserves the comparison of the carboxymethyl-cellulose gel with carboxymethyl-cellulose for chromatography. In spite of the fact that the differences between these two supports are great as regards the yield of the binding and the activity ratio, the K_m-value of amyloglucosidase immobilized on both supports is the same. It seems therefore likely that the decrease in the K_m-value brought about by the binding of the enzyme to the support is caused by specific interactions between the support and the substrate and is not affected by the mode of binding of the enzyme to the support.

The ionogenic groups of the support can significantly affect the pH-optimum of the enzyme[10]. From this viewpoint it is especially important to compare carboxymethyl-cellulose CM-70 for chromatography with the carboxymethyl-cellulose gel; they both have the same structure and bear the same type of ionogenic groups yet differ in the concentration of these groups. Whereas the concentration of these groups in chromatographic cellulose is approximately 1 mmol/g, it is approximately the double in the carboxymethyl-cellulose gel. It may be therefore assumed that this difference will manifest itself in a shift of the pH-optimum of amyloglucosidase bound to these supports. Whereas there is no shift of the pH-optimum of amyloglucosidase bound to the agarose gel compared to soluble amyloglucosidase (Fig. 2), the shift of the pH-optimum of the enzyme bound to chromatographic carboxymethyl-cellulose is approximately 0·6 pH-units and even 1·1 pH-units with the carboxymethyl-cellulose gel. This shift is ascribed to the effect of ionogenic groups[10].

The immobilization of enzymes leads mostly to an increase of their thermal stability[1]. However, the thermal stability of amyloglucosidase did not increase in any of our experiments (Table IV). This can be explained most likely by the relatively high stability of the soluble enzyme which is inactivated relatively slowly even at 70°C.

REFERENCES

1. Mitz M. A., Summaria L. J.: Nature *189*, 576 (1961).
2. Wilson R. J. H., Lilly M. D.: Biotechnol. Bioeng. *11*, 349 (1969).
3. Smiley K. L.: Biotechnol. Bioeng. *13*, 309 (1971).
4. Christison J.: Chem. Ind. (London) *1972*, 215.
5. Arsenis Ch., McCormick D. B.: J. Biol. Chem. *239*, 3093 (1964).
6. Broun G., Thomas D., Gellf G., Domurado D., Berjonaeau A. M., Guillon C.: Biotechnol. Bioeng. *15*, 359 (1973).
7. Cuatrecases P.: J. Biol. Chem. *245*, 3059 (1970).
8. Smiley K. L.: Biotechnol. Bioeng. *13*, 309 (1971).
9. Weibel M. K., Bright H. J.: Biochem. J. *124*, 801 (1971).
10. Katchalski E.: Biophys. J. *4* (No 1, Pt 2) 9 (1964).
11. Silman I. H., Katchalski E.: Annu. Rev. Biochem. *35*, 873 (1966).

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