A monoclonal antibody to cyclomaltoheptaose (β -cyclodextrin): Characterization and use for immunoassay of β -cyclodextrin and its derivatives

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Abstract

Cyclomaltoheptaose (β -cyclodextrin, β -CD) is a promising compound for application in various industrial fields because of its ability to entrap various compounds into its hydrophobic cavity. A monoclonal antibody (A7) to β -CD was generated by using a conjugate of glucosaminylmaltosyl- β -CD and bovine serum albumin as an antigen. The A7 monoclonal antibody was IgM/ κ and reacted with β -CD with high specificity. The epitope recognized by the A7 monoclonal antibody seemed to be located on the secondary hydroxyl groups of the rim side of the β -CD molecule. The dissociation constant of the complex of β -CD and the immobilized A7 monoclonal antibody was determined to be 1.2×10^{-4} M. A competitive ELISA using the A7 monoclonal antibody enabled determination of β -CD and its derivatives with a detection limit of 0.05 μ M. This immunoassay was useful to determine β -CD in biological fluids such as human plasma and urine after appropriate pretreatment of the samples.

Introduction

Cyclomalto-oligosaccharides (cyclodextrins, CDs) are cyclic oligosaccharides composed of glucopyranose units. Three major CDs, α -, β -, and γ -CDs, comprise 6, 7, and 8 glucopyranose units, respectively, and are water-soluble molecules with a torus-like macro ring shape (Szejtli, 1996). Because of their ability to entrap various compounds into their hydrophobic cavity, CDs are useful for improvement of stability, solubility, and reactivity of the entrapped guest compounds. In the food industry (Hashimoto, 1996) CDs are used to stabilize volatile substances (flavors, spices, and essences) and to protect various constituents mixed in food products from the deterioration by oxidation and photochemical reactions. In the cosmetic industry (Hashimoto, 1996), they are used to protect the functional substances in the products from their decomposition and losses through their oxidation

and vaporization. Cyclodextrins entrapping antibacterial substances are used to produce the antibacterial fibers and plastic sheets. Pharmaceutical applications (Uekama, 1996) of CDs are their use as drug delivery vehicles to improve the stability and solubility of drugs, and to target drugs on appropriate tissues or organs.

With the demand for application of CDs in various fields, sensitive and specific methods to detect and quantify CDs have become necessary. Hitherto colorimetry and HPLC with suitable detection systems have been used to quantify CDs (Kubota et al., 1992, 1996; Szenté, 1996). To make a sensitive, specific, and simple assay system of β -CD and its derivatives, an immunoassay using rabbit antiserum (polyclonal antibodies) raised against β -CD was developed (Creminon et al., 1994). Antiserum, however, is an undefined heterogeneous mixture of antibodies which changes with each immunized animal and even

with each bleed of the same animal. A monoclonal antibody produced by an isolated clone is considered as a defined chemical. The permanent culture of the isolated clone makes an unlimited supply of the same antibody possible. Therefore, monoclonal antibodies have been used in various immunochemical analyses in biological and medical fields to identify, isolate, and quantify the antigenic molecules. In this study, we established a murine hybridoma clone that produces a monoclonal antibody against β -CD by using a conjugate of bovine serum albumin (BSA) and β -CD as an antigen. A competitive enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody was shown to be useful for the quantitative analysis of β -CD and its derivatives.

Materials and methods

Materials

The following materials were obtained from the sources indicated: *N*-Acetylchitooligosaccharides (Yaizu Suisankagaku Industry Co., Ltd., Yaizu, Japan), maltosyl-, glucosaminyl-, glucosyl-, galactosyl-, mannosyl-, glucuronylglucosyl-, and *N*-acetylglucosaminyl- β -CDs and some methylated β -CDs (Bio Research Co. Yokohama, Yokohama, Japan), BSA and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, ECDI (Tokyo Chemical Industry Co., Tokyo, Japan), synthetic copolymer adjuvant, TiterMax Gold (CytRx Co., Norcross, GA, U.S.A.), a hybridoma cloning factor, ORIGEN (Igen, Inc., Rockville, MD, U.S.A.), hydroxyapatite (Wako Pure Chemical Industries, Osaka, Japan), an ELISA microtiter plate with 96 wells to fix amino compounds on the surface of wells, Amino-plate, and an ELISA color reagent kit for peroxidase reaction, Type-T (Sumitomo Bakelite Co., Tokyo, Japan), a blocker containing milk proteins, Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan), goat anti-mouse IgG, IgA, and IgM conjugated with horse radish peroxidase (ICN Pharmaceuticals, Inc., Aurora, OH, U.S.A.), a mouse monoclonal antibody isotyping kit (Boehringer Mannheim GmbH, Mannheim, Germany), and Sep-Pak Plus C₁₈ cartridge (Waters Co., Milford, MA, U.S.A.).

Preparation of antigen

To prepare an antigenic substance for the production of antibody to β -CD, glucosaminylmaltosyl- β -CD was covalently bound to BSA by use of

a water-soluble carbodiimide, ECDI, under the conditions described by Waniska and Kinsella (1984). Glucosaminylmaltosyl- β -CD was prepared as follows. *N*-Acetylglucosaminylmaltosyl- β -CD was synthesized from *N*-acetylchitooligosaccharides and maltosyl- β -CD by transglycosylation with lysozyme (Hamayasu et al., 1999). *N*-Acetylglucosaminylmaltosyl- β -CD (1%) was converted to glucosaminylmaltosyl- β -CD by deacetylation under alkaline conditions (10% NaOH, 80 °C, 3 h). After neutralization by 10 M HCl, the deacetylated sample was desalted on an ODS column (Organo, 5 × 100 cm; washed with water and eluted with 30% ethanol), and lyophilized.

Preparation and purification of a monoclonal antibody

Female BALB/c mice (8 weeks old) were immunized by intraperitoneal injection with 0.1 ml of the emulsified antigen solution (5.0 mg ml⁻¹ BSA-glucosaminylmaltosyl- β -CD in a mixture of phosphate-buffered saline (PBS; 10 mM Na-phosphate, pH 7.4/0.15 M NaCl) and TiterMax Gold (1:1)). Two booster injections were given in the same way at 2-week intervals. The second booster injection was given 3 days before cell fusion. The fusion of spleen cells from the immunized mouse and mouse myeloma cells (SP2/O-Ag14) by use of polyethylene glycol-1500 and hybridoma selection culture using a medium containing hypoxanthine, aminopterin, and thymidine were done as described previously (Ikura et al., 1984a), except that Dulbecco's modified Eagle's medium was used as a base medium instead of RPMI-1640 medium. Hybridoma cultures producing antibodies that bound to β -CD were selected by ELISA using glucosaminyl- β -CD as an immobilized antigen. Hybridoma cells from cultures positive for the antibody production were cloned by the limiting dilution method as described previously (Ikura et al., 1987), except that a hybridoma cloning factor, ORIGEN, was added (10%) to the diluted culture instead of mouse thymocytes used as feeder cells.

The ascitic fluid of BALB/c mice containing the monoclonal antibody to β -CD was obtained as described previously (Ikura et al., 1984b). The monoclonal antibody in the ascitic fluid (10 ml) was precipitated by adding an equal volume of a saturated ammonium sulfate solution to the ascitic fluid diluted with an equal of PBS and by subsequent centrifugation. The precipitated antibody sample was dissolved in 10 ml of 10 mM Na-phosphate buffer (pH 6.8)

(Buffer A) and dialyzed against the same buffer. After centrifugation of the dialysate, the supernatant was applied on a hydroxyapatite column (3.5×12 cm) equilibrated with Buffer A. Following a washing the column with Buffer A (530 ml) and 150 mM Na-phosphate buffer (pH 6.8) (420 ml), the antibody fraction adsorbed to the column was eluted with 250 mM Na-phosphate buffer (pH 6.8) (220 ml). To this eluted fraction, solid ammonium sulfate was added to 75% saturation and the precipitated antibody was obtained by centrifugation. The precipitate was dissolved in and dialyzed against PBS. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970) and densitometric analysis indicated that more than 95% of the protein in the dialysate was mouse IgM. To the dialysate, the protein concentration of which was 2.1 mg ml^{-1} , we added sodium azide (0.02%) and stored it at -80°C in small aliquots.

All procedures involving mice were carried out in accordance with the animal care guidelines established by Japanese government and Kyoto Institute of Technology.

ELISA

Procedures were at room temperature unless otherwise indicated. Amino-plates, plastic microtiterplates with 96 wells which were chemically treated to fix amino compounds covalently on the surface of the wells by using glutaraldehyde as a linker, were used for ELISA to detect antibodies that react with β -CD. To each well of the plates was added $100 \mu\text{l}$ of 2% glutaraldehyde. The plates were incubated at 30°C for 2 h and wells were washed with water. To each well was added $100 \mu\text{l}$ of a 0.15 mg ml^{-1} glucosaminyl- β -CD solution in PBS. The plates were incubated at 4°C overnight. After the solution was removed, $100 \mu\text{l}$ of the mixture of Block Ace and PBS (1:3) was added to each well. The plates were incubated at 30°C for 2 h and wells were washed with PBS containing 0.05% Tween 20 (washing buffer). Portions ($50 \mu\text{l}$) of antibody samples were added and the plates were incubated at 30°C for 2 h. After the wells were washed with the washing buffer, $50 \mu\text{l}$ of $4 \mu\text{g ml}^{-1}$ goat anti-mouse IgG, IgA, and IgM conjugated with peroxidase in the washing buffer was added to each well and the plates were incubated at 30°C for 1 h. After the wells were washed with the washing buffer, $100 \mu\text{l}$ of the peroxidase reaction mixture was added to each well. After incubation at 30°C for 20 min in the dark, the

peroxidase reaction was stopped by addition of the stopper solution ($100 \mu\text{l}$) of the kit reagents, and the absorbance of each well was measured at 450 nm.

Competitive ELISAs were done in the same way as the standard ELISA described above, except that the sample antibody solutions contained different amounts of a competitor compound together with a constant amount of a monoclonal antibody. Results were given in terms of $A/A_0 \times 100$ (%) as a function of the competitor concentration (Figure 1), here A and A_0 represent the absorbance in the presence and absence of competitor, respectively. The cross-reactivity of the competitor was shown by the concentration of competitor lowering 50% the absorbance observed in the absence of competitor (A/A_0 50%). The lower the value of this index, the higher the cross-reactivity of the competitor is. The relative cross-reactivity (RCR) of competitor was based on the cross-reactivity of β -CD and shown by $((A/A_0 \text{ 50\% of } \beta\text{-CD})/(A/A_0 \text{ 50\% of competitor}) \times 100$ (%).

Analysis of a dissociation constant

An automatic analyzer of interaction between biomolecules, FAST IAsys Cuvett System (Affinity SENSORS, Cambridge, U.K.), was used to determine the dissociation constant of the complex of a soluble β -CD and an immobilized monoclonal antibody A7. The monoclonal antibody A7 was immobilized to the carboxylated sensor surface of the IAsys cuvette according to the manufacturer's protocol by using 0.2 ml of antibody solution (0.9 mg ml^{-1} in PBS). The binding profiles recorded at each β -CD concentration were analyzed by using a software of FASTfit.

Results and discussion

A monoclonal antibody specific to β -CD

Eight hybridoma clones that produced monoclonal antibodies reactive with β -CD were obtained from three positive hybridoma cultures prepared by two cell fusions between spleen cells from mice immunized with BSA-glucosaminylmaltosyl- β -CD conjugate and mouse myeloma cells. The class of all immunoglobulins produced by these eight hybridoma clones was IgM and their light chain was all κ . From these eight clones, one clone (named A7) was selected to be a useful clone for this study, because A7 clone grew better and produced a monoclonal antibody more efficiently than seven other clones. To confirm the specific

Figure 1. Competitive ELISA showing specific reactivity with β -CD. A7 antibody was used at the concentration of $0.08 \mu\text{g ml}^{-1}$ in PBS. The concentration of the competitor (β -CD) was changed from 0.005 to $5.0 \mu\text{M}$. The abscissa indicates the competitor concentration in logarithmic scale. The relative absorbance at 450 nm was based on the absorbance obtained in the absence of the competitor.

cross-reactivity of the A7 monoclonal antibody to β -CD, we performed the competitive ELISA, in which the inhibitory effects of the β -CD and its analogs and derivatives on the binding of the monoclonal antibody to the fixed β -CD were tested (Table I). β -CD repressed the reaction between the monoclonal antibody and the fixed β -CD in a dose-dependent manner with the value of A/A_0 50% of $0.25 \mu\text{M}$ (Figure 1). The values of RCR of α -CD (cyclomaltohexaose) and γ -CD (cyclomaltooctaose) were 8.9 and $<0.1\%$, respectively. These results indicated that, compared with β -CD, α -CD was slightly recognized by the A7 antibody and γ -CD was hardly recognized. This suggested that the addition of one D-glucose unit to the cyclic structure of β -CD caused a larger structural change of the epitope of β -CD than the deletion of that. β -CD derivatives in which various kinds of residues (glucosyl, maltosyl, galactosyl, mannosyl, glucuronyl-glucosyl, glucosaminyl, *N*-acetylglucosaminyl, and glucosaminylmaltosyl residues) were introduced to the hydroxyl group of one of seven C-6 positions, showed the cross-reactivity with the A7 antibody comparable with that of unmodified β -CD. These results indicated that the modification of one of seven carbons of C-6 in β -CD molecule did not change so much the structure antigenic to the A7 antibody. The linear malto-oligosaccharides (triose, tetraose, pentaose,

hexaose, and heptaose) were poorly recognized by the A7 antibody, indicating that the cyclic structure constructed by seven glucose units is important in forming the epitope structure of β -CD. A slight cross-reactivity exhibited by the pentamer, hexamer, and heptamer of glucose suggest that these malto-oligosaccharides may have curved structures of glucose chains more similar to those of α - or β -CDs than that of γ -CD.

Epitope region of β -CD molecule

To search for the epitope region of β -CD molecule recognized by the A7 antibody, the cross-reactivities of several β -CDs methylated at different sites of hydroxyl groups were compared (Table I). The β -CD methylated at all sites of C2 and/or C3 (heptakis (2-*O*-methyl), heptakis (3-*O*-methyl), heptakis (2,3-*O*-dimethyl), heptakis (2,6-*O*-dimethyl), heptakis (2,3,6-*O*-trimethyl) β -CDs) exhibited almost no cross-reactivity, while the β -CD methylated at only C6 sites (heptakis (6-*O*-methyl) β -CD) maintained the cross-reactivity comparable with that of unmethylated β -CD. These results indicated that the epitope region recognized by the A7 antibody probably is located at the secondary hydroxyl rim of β -CD molecule with a torus-like macro ring shape. This may be related to the fact that BSA-glucosaminylmaltosyl- β -CD and glucosaminyl- β -CD, in which C6 rim site of β -CD is

Table 1. Relative cross-reactivity of β -CD analogs and derivatives, and maltooligosaccharides in competitive ELISA

Competitor compounds	A/A ₀ 50% ^a (μ M)	RCR ^b (%)
β -CD	0.25	100
α -CD	2.80	8.9
γ -CD	>200	<0.1
Glucosyl β -CD ^c	0.34	74
Maltosyl β -CD ^c	0.54	46
Galactosyl β -CD ^c	0.36	69
Mannosyl β -CD ^c	0.31	81
Glucuronylglucosyl β -CD ^c	0.30	83
Glucosaminyl β -CD ^c	0.53	47
N-Acetylglucosaminyl β -CD ^c	0.83	30
Glucosaminylmaltosyl β -CD ^c	0.59	42
Heptakis (2- <i>O</i> -methyl) β -CD	>500	<0.1
Heptakis (3- <i>O</i> -methyl) β -CD	>500	<0.1
Heptakis (6- <i>O</i> -methyl) β -CD	0.46	54
Heptakis (2,3- <i>O</i> -dimethyl) β -CD	>500	<0.1
Heptakis (2,6- <i>O</i> -dimethyl) β -CD	>500	<0.1
Heptakis (2,3,6- <i>O</i> -trimethyl) β -CD	>500	<0.1
Maltotriose	>500	<0.1
Maltotetraose	420	<0.1
Maltopentaose	36.0	0.7
Maltohexaose	15.6	1.6
Maltoheptaose	16.5	1.5

^a Cross-reactivity: a concentration of the competitor compound lowering 50% the absorbance observed in the absence of the competitor compound.

^b Relative cross-reactivity: this was based on the cross-reactivity of β -CD and shown by (A/A₀ 50% of β -CD)/(A/A₀ 50% of competitor compound) $\times$ 100 (%).

^c In these β -CD derivatives, each residue is introduced to the hydroxyl group of one of seven carbons at the C-6 position.

oriented to the adjacent linker molecules, were used as an antigenic substance of β -CD for immunization and in ELISA to detect the antibody reactive to β -CD, respectively.

Dissociation constant of β -CD and A7 antibody complex

A dissociation constant of the complex of β -CD and the A7 antibody immobilized on the surface of sensor cuvette was determined by using the FAST IAsys Cuvette System. Figure 2 shows a plot of k_{on} (the binding-rate constant for particular concentration of β -CD) against β -CD concentration. The association

Figure 2. Association plot for the interaction of soluble β -CD with the immobilized A7 antibody. Data for the plot were obtained by using a computing software FASTfit for the FAST IAsys System analyzer. k_{on} : binding rate constant.

rate constant, k_{ass} (the gradient: 8.0 ± 2.2), and the dissociation rate constant, k_{diss} (the extrapolated intercept on the Y-axis: 0.0010 ± 0.00034) were obtained from this plot. The dissociation constant equivalent to k_{diss}/k_{ass} was calculated to be 1.2×10^{-4} M. The reason why the dissociation constant was so high is not clear. The immobilization of the A7 antibody on the surface of sensor cuvette possibly lowers the reactivity of the antibody. However, the obtained value is consistent with the report (Sharon et al., 1982) that the binding constants with isomaltoheptaose of monoclonal BALB/c mouse IgM antibodies specific for $\alpha(1 \rightarrow 6)$ linked dextran were from 6.5×10^3 to 7.7×10^4 M⁻¹, which were determined by affinity electrophoresis method.

Immunoassay of β -CD and its derivatives

By using the plot of the competitive ELISA with the A7 monoclonal antibody as a standard curve, β -CD could be determined readily at a range of concentrations from 0.05 to 1.0 μ M in the assay system (Figure 1). In the same competitive ELISA system, the β -CDs derivatives (glucosyl, maltosyl,

Figure 3. Standard curves obtained from the data of competitive ELISA using β -CD dissolved in human plasma (A) and urine (B) as competitors. Competitors are: (●) β -CD in PBS, (○) β -CD in plasma (Sample was mixed with 1/5 volume of 0.5 M perchloric acid and the appeared precipitate was removed by centrifugation. This was repeated two more times and the finally obtained supernatant was neutralized with 0.5 M K_2HPO_4), (▲) β -CD in urine (Sample (3.0 ml) was put into a reverse-phase Sep-Pak Plus C_{18} cartridge, washed with 9.0 ml of water, and eluted with 3.0 ml of 8% acetonitrile. The eluted β -CD fraction was lyophilized and dissolved in PBS).

galactosyl, mannosyl, glucuronylglucosyl, glucosaminyl, *N*-acetylglucosaminyl, and glucosaminylmaltosyl β -CDs) also could be determined with a sensitivity similar to that of β -CD (Table I, data not shown). The detection limit of 0.05 μ M in our ELISA system corresponds to 2.5 pmol. This detection limit is comparable with the detection limits (around 5 pmol) of high

sensitive HPLC methods using a pulsed amperometric detector (Kubota et al., 1992; Szente, 1996).

Immunoassay of β -CD in biological fluids

A possibility of the immunoassay for β -CD in biological fluids (human plasma and urine) was examined. Plasma (at concentrations of higher than 0.5%) and

urine (at concentrations of higher than 15%) strongly repressed the immunoreaction in ELISA with the A7 monoclonal antibody and lowered the sensitivity and accuracy of the competitive ELISA (data not shown). However, by doing appropriate pretreatments such as the deprotenization of the plasma by perchloric acid and separation of β -CD fraction from urine by reverse-phase C₁₈-cartridge (see legend of Figure 3), the standard curves for the immunoassay of β -CD in human plasma and urine could be drawn in the same manner as β -CD in PBS (Figure 3). Some proteins at high concentrations in plasma and hydrophobic components in urine seemed to interfere with the immunoreaction in ELISA.

Conclusions

By the cell fusion method, one mouse hybridoma clone that produced a monoclonal antibody (A7) to β -CD was established. The A7 monoclonal antibody reacted with β -CD with high specificity. The competitive ELISA using various β -CD derivatives indicated that an epitope recognized by the A7 monoclonal antibody is located on the secondary hydroxyl groups of the rim side of the β -CD molecule. By using the plot of the competitive ELISA with the A7 monoclonal antibody as a standard curve, β -CD and its derivatives could be readily determined. β -CD in human urine and plasma also could be determined without a decrease of the assay sensitivity by appropriate pretreatment of samples. These results indicated that the obtained A7 monoclonal antibody to β -CD may be a useful tool to detect β -CD quantitatively and qualitatively in its application studies of various fields.

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