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α-MANNOSIDASE ACTIVITY FROM ANTIBODY RAISED AGAINST A GLUCAL ANTIGEN

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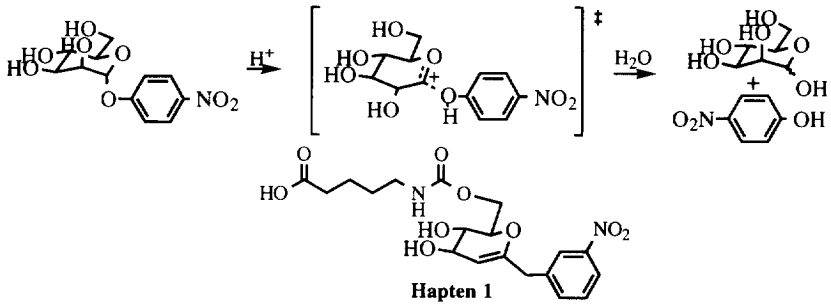
Abstract: Sixty cell lines of monoclonal antibody were raised against a glucal hapten **1**. Among them, Ab 405.4 showed α-mannosidase activity as k_{cat} = 0.19 /day (k_{cat}/k_{uncat} = 110,000). The chemical modification study and pH profile study of this antibody indicated that carboxyl group(s) in the antigen binding site involved in catalytic mechanism.

Since catalytic antibodies are supposed to have catalytic activities with tailor-made specificities, efforts to generate these antibodies are through mimicry of various natural enzymes. For example, production of catalytic antibodies for glycosidase had been initiated by Lerner's,[1] Massamune's,[2] and Schultz's group,[3] independently. Recently, genuine glycosidase antibodies were generated using a phage display library[4] and *in vitro* immunization method.[5] By developing and studying these antibodies, we might expend our knowledge of structure and activity of the related enzymes.[6] One of the advantage of antibody catalyst over natural glycosidase is its specificity. In this respect, catalytic antibody could be substitutes for the natural glycosidases. By developing such glycosidase antibodies with enhanced substrate specificities, building a restriction enzyme system for glycosidase would be possible. We have been puzzled, however, that only β-anomer specific antibodies were isolated in the previous reports.[4-5] The transition state analogues used in those studies must have resembled more of β-glycosidic bond than α-glycosidic one. Here, we successfully produced the catalytic antibody for α-mannosidase activity using a different transition state analog, a glucal hapten **1**, and report it here.



Hapten 1

At the transition state on glycosidic bond cleavage, pyranoside ring is known to have a twist or twist-boat conformation due to the partial double bond.[7] On the basis of this geometric characteristic, we designed glucal hapten **1** as a transition state analog of glycosidic bond cleavage.[8] Hapten **1** was synthesized from tri-O-acetyl glucal in eight steps with 8% overall yields.[3] The carboxyl group in the hapten was converted to the activated ester to attach the hapten to the carrier protein (Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA).[3] The immunization of KLH conjugated hapten **1** and generation of antibodies were done as previously

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described.[9] Sixty cell lines of antibodies were selected by enzyme linked immunosorbent assay (ELISA), and ascites from each cell line were generated. The ascites were purified using Protein-A affinity chromatography followed by an ion-exchange chromatography employing mono-S column.[10] Initially, antibodies were assayed using *p*-nitrophenyl-α and β–D-glucopyranosides as substrates, without any catalytic activity.[3] However, we further tried to assay the catalytic activity using *p*-nitrophenyl-α and β–D-galactopyranoside and *p*-nitrophenyl-α and β–D-mannopyranoside to find enhanced substrate specifities, because the hydroxyl orientation of the glucal hapten might not be enough to define glucopyranosides as the most specific substrare for the generated antibodies.[11]

Five cell lines of antibody were found to be catalytic. Ab 405.4 showed the best catalytic activity for *p*-nitrophenyl-α–D-mannopyranoside and was chosen for further studies. This antibody showed a saturation kinetic and its enzymatic constants were determined: K_m = 1.0 mM, k_{cat} = 0.19 /day (k_{cat} /k_{uncat} = 1.1 x 10^5).[12] As the K_m value of the antibody indicated, the binding of the specific substrate is moderate, due to the chair conformation of the substrate, while the conformation of the hapten is a twist or a twist-boat. We defied the possibility of the catalytic activities coming from contaminating natural enzymes, not our antibodies based on the following observation. First, we had 55 negative control cell lines of antibodies that were raised against the same hapten but they did not show contaminated glycosidase activity. Second, after absorbing catalytic antibodies with Protein-A agarose gel, resulting supernatants showed decreased catalytic activities to the same magnitude of the reduced amount of antibodies.[13] Finally, the whole antibody was digested with papain and Fab was purified by an ion exchange chromatography using mono S column.[14] The purified Fab fraction (major one) showed the retained α-mannosidase activity while the other fractions gave no activity at all, suggesting Fab is only source of the catalytic activity.

α–Mannosidase activity of Ab 405.4 was partially inhibited by the hapten. The K_i value of Ab 405.4 was determined to be 220 μM at pH 4.5, by Dixon plot in **Figure 1 (a)**, indicating moderate affinities between hapten **1** and the antibody. pH Dependence study of the catalytic activity of the antibody was performed in

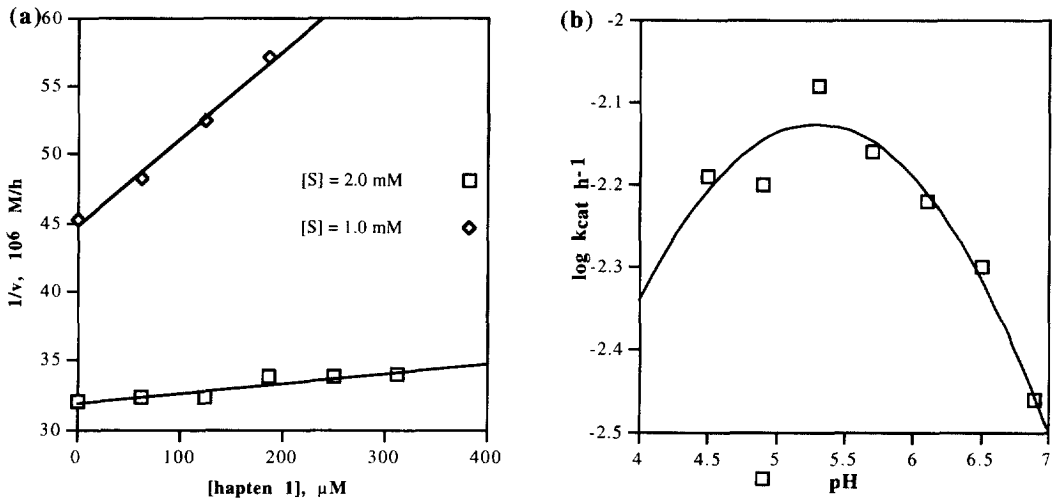

Figure 1. **(a)** Dixon Plot of Ab 405.4 by the Hapten **1**. 2.0 mM (■) and 1.0 mM (◆) of substrate solution used. **(b)** pH Profile of Ab 405.4. See footnote[11] for the experimental details.

Figure 1 (b), showing maximum k_{cat} at pH 5.3, indicating that some carboxyl residues in the antigen binding site are involved in the catalytic mechanisms as general acid. Chemical modification study on Ab 405.4 by diazoacetamide showed 70% activity reduction,[15] confirming the carboxyl group(s) involvement in the catalytic mechanism. Considering past study that showed no correlation between catalytic activity and the affinity to haptens in conventional *in vivo* immunized antibodies,[16] our results in this study made us to believe that the low affinity antibody does not interfere with catalytic activities. It is a bit strange that the α-glucopyranoside was not a substrate but α-mannopyranoside was. However, equatorial orientation of 2-hydroxyl group in α-glucopyranoside is absent in the glucal hapten, suggesting glucopyranoside is not necessarily a plausible substrate. In our results, the specificity was not confined to the equatorial orientation but happen to be confined to the axial orientation in the complimentary structure of Ab 405.4, allowing α–mannopyranoside the most specific substrate. Cloning and sequencing of the antibody genes from the hybridoma cell line and subsequent mutation studies is under progress to understand the detailed catalytic mechanisms and substrate specificity and to improve catalytic activity with compatible k_{cat} to the natural glycosidases.

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10. The SP-Separose® (HiLoad™ 16/10) column (Pharmacia) was used for the stationary phase. For the mobile phase, buffer "A" (50 mM MES at pH 5.5) and buffer "B" (50 mM MES, 1.0 M NaCl at pH 5.5) were co-eluted. Antibodies were usually eluted at 30-35% of buffer "B".
11. To 90 μL of antibody solution (2-3 mg/mL, 10 mM of N-(2-morpholino)ethansulfonic acid (MES), 100 mM of NaCl, 0.02% of sodium azide at 37°C at pH 4.5.), 10 μL of 20 mM (same buffer) substrate solution was added. The resulting mixture was incubated at 37°C for 3 days. The activity was measured by injecting 10 μL of this incubated mixture into HPLC in every 24 h, and monitoring at 315 nm. A C18 column (Microsorb; 4.6 mm x 15 cm, 5 μm) with a guard module (4.6 mm x 2.5 cm) was used as a stationary phase. Isocratic run of 50% methanol with 0.8 mL/min gave 5.7 min retention time for *p*-nitrophenol. The

amount of *p*-nitrophenol in the injected solution was determined both by peak height and integration unit. The rate was determined when less than 1% of the substrate was converted to the product.

12. The background hydrolysis of *p*-nitrophenyl-α-D-mannopyranoside was performed at the same conditions as described in footnote.[11] The second order rate constants for the [H^+] and [OH^-] catalyzed reactions and the pseudo-first order rate constant for the [AcOH] and [H_2O] catalyzed reactions are: k_{H+} = 2.8 x 10^{-3} $M^{-1}h^{-1}$; k_{OH-} = 2.8 $M^{-1}h^{-1}$; k_{AcOH} = 3.1 x 10^{-6} $M^{-1}h^{-1}$; and k_{H2O} = 7.1 x 10^{-8} h^{-1}. pH Dependence of background hydrolysis rates was shown in **Figure 2**.

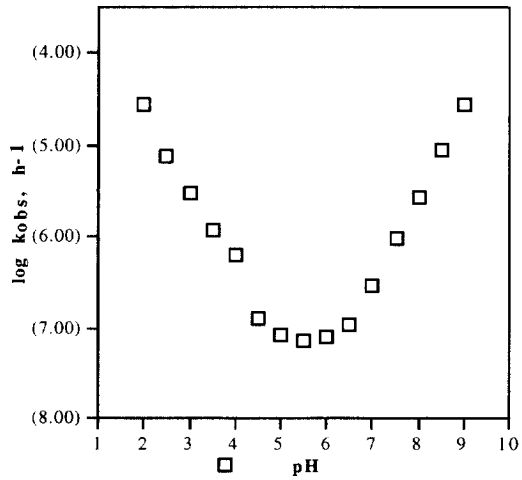

Figure 2. pH Dependence of the hydrolysis rates of *p*-nitrophenyl-α-D-mannopyranoside (pseudo-first order reaction constant, k_{obs}). The reaction temperature was 37°C for all reactions. The buffer salts used were HCl/acetate, acetate, MES, N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid (EPPS), N,N-bis-(2-hydroxyethyl)glycine (BICINE) for pH 2-3, 3-4.5, 4.5-7, 7-8, 8-9, respectively. Ionic strength was adjusted to be 100 mM NaCl.

13. A solution of antibodies (1-2 mg/mL) in the assaying buffer was add to the dry protein-A agarose gel. The resulting solution was gently stirred for 30 min and the gel was filtered out. The resulting filtrate was incubated with the substrate at 37°C for 24 h, showing activity decrease, which is the same amount of antibody decrease in solution. The filtered agarose gel was re suspended in the substrate solution and incubated at 37°C for 24 h, and showed glycosidase activities.
14. The whole antibody (IgG) was digested with papain as was suggested in Parham, P. *J. Immunol.* **1983**, *131*, 2895. The digested Fab soluton was ion-exchange chromatographed as was refered in 10. The Fab was eluted at 20% of buffer "B".
15. To a solution of antibody (1 mg/mL) in the presence and absence of hapten **1** (2 mM), 200 volumes of 0.4 M diazoacetamide solution (150 mM $NaClO_4$, 20 mM boric acid, pH 8.0) was added and stirred for 8 h at room temperature. The resulting solution was dialyzed in the assaying buffer (10 mM MES, 100 mM NaCl; pH 4.5) and concentrated for the assay. Grossberg, A.L. Pressman, D. *J. Am. Chem. Soc.* **1960**, *82*, 5478.
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