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Polyclonal Antibody-Catalysed Aldimine Formation

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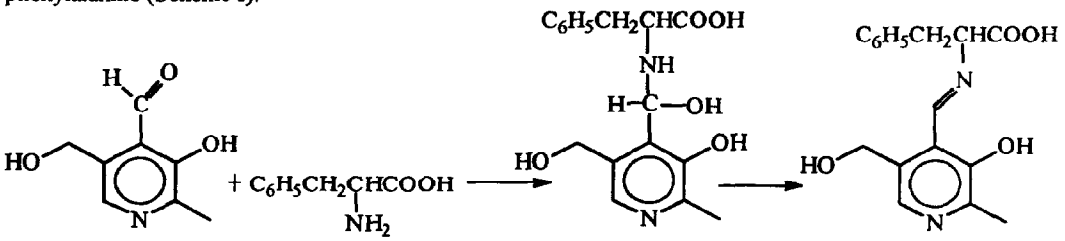
Abstract: An antiserum catalysed imine formation from pyridoxal and phenylalanine is described, under conditions in which the uncatalysed reaction is not observed. This polyclonal antibody preparation shows Michaelian kinetic characteristics and presents a very good specificity for the pyridoxal structure.

The use of catalytic antibodies has been rapidly increasing since 1986[1] when the powerful potential of monoclonal antibodies was demonstrated. At that time polyclonal antibodies were thought to be useless since all attempts to found any catalytic effect were unfruitful.[2] However, since 1988 it has been recognised that such polyclonal antibodies can display some catalytic activity and that they have some advantages over monoclonal antibodies.[3,4,5] They are easier to prepare and they open the way to the investigation of catalytic antibody responses in animals, which is of great importance for the development of novel therapies.[6]

Furthermore if it should be possible to observe a catalytic effect by using directly the antiserum of immunised animals, without isolation of the antibodies, it could be very useful for the development of new catalysts directed toward organic synthesis.

In order to study such an approach, we chose as a model reaction the condensation of pyridoxal and phenylalanine because monoclonal antibodies catalysed aldimine formation between 5'-deoxy pyridoxal and p-nitrophenylalanine has been previously described.[7]

We report here the preparation and the catalytic activity of a polyclonal antibody preparation which fulfils the above requirements and catalyses the bimolecular aldimine formation between pyridoxal and phenylalanine (Scheme I).

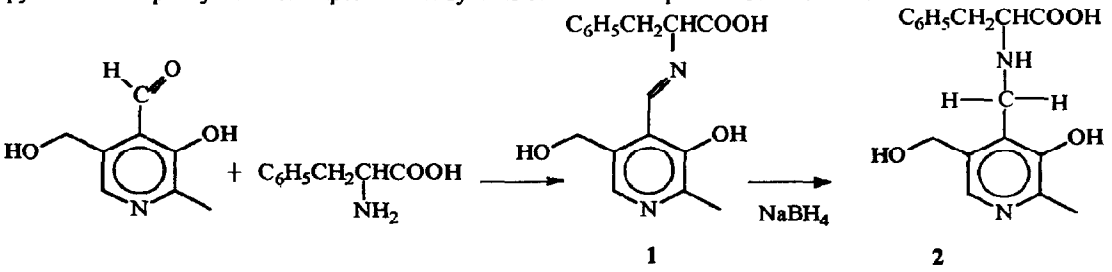


Scheme 1

Such a reaction is of great importance as it represents the first step of many biological transformations of aminoacids involving the enzymatic cofactor pyridoxal phosphate (PLP) (e.g. transamination, decarboxylation, racemisation, β,γ-elimination, ethylene biosynthesis...).[8] In these reactions the amino group of an amino acid attacks the imino function of the adduct between an enzyme and PLP. Formally this imino function can be seen as the masked carboxaldehyde function of PLP.

As the transition state of the first step of the reaction should be very similar to the tetrahedral

intermediate involved, hapten 2 was designed to mimic the transition state of the reaction between pyridoxal and phenylalanine. Hapten 2 was synthesised in two steps as described in Scheme II.



Scheme II

The aldimine 1 was obtained in good yields by base-catalysed condensation of pyridoxal and D,L-phenylalanine and then reduced with sodium borohydride to give hapten 2.[9] The immunogen was prepared by coupling hapten 2 with Bovine Serum Albumin (BSA) via the N-hydroxy succinimidyl ester produced in situ with 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide activation.

Four rabbits were immunised with 100 µl of the immunogen in solution (3.75 mg per ml of phosphate- buffered saline solution: NaCl 0.15M, HNa_2PO_4 10^{-2}M, pH 7.0). This was repeated at four three weekly intervals. The animals were bled ten days after each re-immunisation. The blood was centrifuged (2000g) and the antiserum was stored at -20°C. It was used as such.

The four antisera appeared to catalyse the reaction between pyridoxal and D,L phenylalanine. We checked that the effects are actually due to antibodies and not to an induced enzymatic catalysis[2]: antibodies to rabbit immunoglobulins were linked to Affigel (Bio Rad); the gel was then incubated with antiserum (overnight, 4°C) and washed. The rabbit immunoglobulins immobilised onto the gel did show a catalytic effect. After acid treatment of the later, antibody concentration in the eluate was estimated by using ε_{280}=2. $10^5 M^{-1}$ cm^{-1} and was approximatively 0.20µM [3b]

Figures 1 and 2 display the results obtained with the best antiserum ATB3.

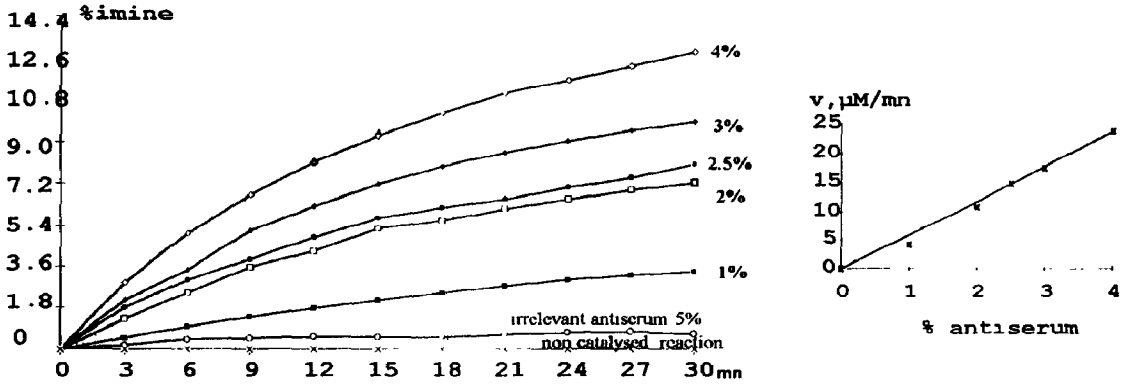


Figure 1: Influence of antiserum concentration on imine formation .

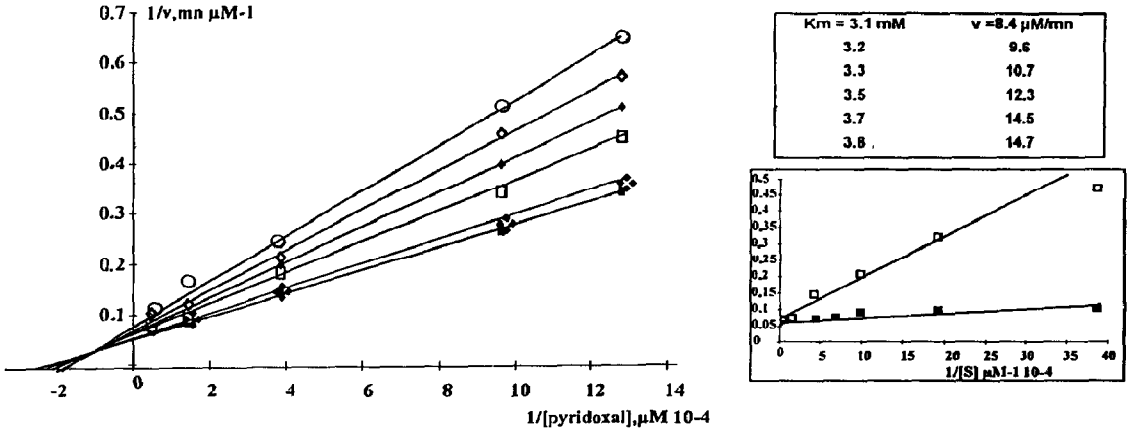


Figure 2 : Lineweaver-Burk plots for imine formation as a function of [[pyridoxal] at various six concentrations of D/L-phenylalanine :○130µM, ◊ 260 µM,◆ 520 µM,□ 1.04 mM, ❖ 1.5 mM and ■ 2.6mM , 3% antiserum.
Inserts · -Kmpyr and Vm
-replot y-intercepts of Lineweaver-Burk plots as a function of [pyridoxal] (□) 260 µM, 520µM, 1.04 mM, 2.6mM, 7.2mM, 20 mM and [phenylalanine](■)

The reaction was monitored by UV spectroscopy, measuring the differential absorption between 430 nm (near the λ_{max} of the imine : 410 nm) and 500 nm (no absorption for the imine) in NaCl solution (5.10^{-2} M) buffered with HNa_2PO_4 (5.10^{-2}M) at pH 7.6. In a typical experiment, 0.39 µM of pyridoxal was allowed to react with 0.39 µM of phenylalanine and 0 to 4 % of antiserum, in 1.5 ml of the buffer solution. All reactions were performed at 21°C. The imine was characterised after reduction to amine 2 (sodium borohydride) by HPLC (comparison with an authentic sample of 2); its structure was confirmed by ^1H NMR spectroscopy.

As it can be seen on Figure 1 the antiserum shows Michaelian kinetic characteristics. The catalytic effect is dependent on antiserum concentration: between 1% and 4% a linear correlation is observed for initial rates (Fig.1, insert). As shown by the results of blank experiments, in the absence of antiserum or with irrelevant antiserum, almost no reaction is observed between pyridoxal and phenylalanine.

It must be realised that, in our conditions, the aldimine formation is in competition with its own hydrolysis and that the observed effect does not represent the real acceleration of imine formation but the result of the two competitive processes: imine formation and imine hydrolysis. Indeed it was shown that the rate of imine hydrolysis leading to the starting compounds was slightly lowered when the hydrolysis was realised in presence of antiserum ATB3.

The polyclonal antibody catalysed reaction is inhibited when 0.48 µM of hapten 2 is added to a mixture of pyridoxal (0.39 µM), phenylalanine (0.39 µM) and 3% of antiserum. With only 1% of antiserum, total inhibition was observed with 0.39 µM of haptene 2.

The activity of antibodies is not much affected by temperature; for instance, a loss of only 10% of the

activity was observed when antibiodies were incubated at 40°C for 14 hours.

The initial velocity data at six fixed concentrations of racemic phenylalanine (1.5 mM: saturant concentration[10]) and varied concentrations of pyridoxal yield a family of Lineweaver-Burk plots (Figure 2). After demonstration of adherence of the corrected *v*-versus-[pyridoxal] data to the Michaëlis-Menten equation by the linearity of a [pyridoxal]/*v*-versus-[pyridoxal] plot, y-intercepts of Lineweaver-Burk plots as a function of [pyridoxal] and [phenylalanine] were plotted (Figure 2 insert)[11] and values of the parameters : V_{max} (15μM mn^{-1}), K_{mpyr} (3 9mM) and K_{mphe} (160μM) were inferred Because of the high value of the saturant concentration of pyridoxal the experimental determination of the corresponding kinetic parameters was not possible [10]

A kinetic analysis of aldimine formation from L- or D-phenylalanine in the presence of ATB3 indicated specific catalysis with both D- or L-isomers. In the two sets of experiments the values for K_m and V_{max} were very similar. The same study realised with 2-, 3- or 4- pyridine carboxaldehyde instead of pyridoxal showed that there is no catalytic effect with these substrates This indicates that the polyclonal antibodies present a very good specificity for the pyridoxal framework.

Our results show that polyclonal antibodies can not only catalyse hydrolysis reactions, as previously described, but also bimolecular condensation reactions for which only one example was previously reported for a photochemical reaction.[5] Furthermore and more important for chemical applications the antiserum can be used as such in the model reaction described. As these antiserum are very easy to obtain, they offer interesting perspectives for new catalysed reactions in organic synthesis.

Further work is presently in progress to extend this approach to more elaborate organic reactions and to enantioselective reactions by using optically active immunogens.

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