the 6-phospho-ß-galactosidase from *Staphylococcus aures*, is thought to be acid/base catalyst because of the 1000-fold reduction in k_{cat} and 200-fold reduction in k_{cat}/K_m for the hydrolysis of *p*NPGal by the E160N mutant (39). In addition, the equivalent residue of a Family 1 enzyme from Cassava was identified by tagging with the affinity label N-bromoacetyl-ß-D-glucopyranosylamine and was suggested to be the acid-base catalyst (40).

The substitution of E in TFNEP region with Q in *Glu1* and *Glu2* aimed to mimic catalytic site of myrosinase from *Synapsis alba*. Myrosinase contains Q instead of E (acid/base catalyst) in proposed acid/base catalyst TFNE/QP region, which provides protonation assistance to aglycone departure in all O-ß-glucosidases. The Q mutant showed substantial activity towards *p*NPG and *o*NPG. Moreover, it has been shown that there is no any other residue positioned near the TFNQP region, which may provide proton for the hydrolysis (32). Therefore, it has been the subject of considerable debate how myrosinase provides proton for hydrolysis. Burmeister et al. (32) discussed that the sulfate group of the substrate is considered to assist this function making the aglycone as a good leaving group. This shows that S-glucosidase, myrosinase, obtains the proton from sulfate group of the substrate due to low pK_a of sulfate for catalysis. In the 3-D structure of the enzyme-glucone complex, Q occupies a position within the active site equivalent to that of the acid-base catalyst E of O-glycosidases (32). Wang and Withers (34) and Burmeister et al. (32) proposed that the hydrolysis of ß-glucosinolates by myrosinases does not require an acid-base catalyst because glucosinolate aglycones are good leaving groups. Similarly, the deglycosylation step does not require a base catalyst E because Q in the myrosinase active center positions a water molecule in such a way that it can hydrolyze the covalent bond between glucose and the enzyme. However, in this scheme, the source of the proton needed for aglycone departure in the glycosylation step is not known. However, it has been reported that myrosinase is able to hydrolyze *p*NPG in a low efficiency. It is necessary to provide a proton for the hydrolysis of *p*NPG. The *p*NPG does not contain a functional group like sulfate of sinigrin, which assists to break the glycosidic S-linkage. This suggests that water molecule is activated by glutamate residue in myrosinase which is believed to hydrolyze glucosinolate substrates without base catalyst. Our experimental results clearly showed that Q replacement in position 191 of Glu1 does not enable the mutant to hydrolyze sinigrin substrate and synthetic *p*-nitro-phenyl-thio-ß-D-glucospyranoside, which is an inhibitor of Glu1 (Table II). This

suggests that there is specific topological arrangement in the active site of myrosinase that assists the aglycone moiety of sinigrin to depart in the glycosylation step.

Addition of exogenous nucleophiles such as azide was reported to restore the activity of the *Agrobacterium* mutant in which the general acid/base residue has been changed to glycine (30). In contrast, addition of azide as nucleophile to the reaction mixture did not significantly enhance the activity of maize E191Q mutant (data not shown). This difference may be steriochemistry of the substituting residues, because the glycine may leave space for the access of small nucleophiles to the active site whereas the larger glutamine may not.

The back mutation of the E191Q (EQ191E) mutants of the Glu1 and Glu2 isozymes to wild type restored full catalytic activity in both cases as shown by non-denaturant 6% activity gel staining with 4MUGlc (Fig. 3). This provides proof that the general acid/base catalyst, E191, of maize ß-glucosidases has been removed and the activity loss can not be attributed to any other spontaneous mutation within the active site structure of the enzymes. However, after normalizing *p*NPG activity of wild type Glu1 and E191Q mutant, hydrolysis of 4MUGlc by E191Q mutant was clearly shown in non-denaturant 6% activity gel (Fig. 4). In addition, the physiological substrate DIMBOAGlc hydrolysis by Glu1 and E191Q mutant was shown in TLC (Fig. 4). Comparative densitometric analysis of glucose in TLC indicated that Glu1 hydrolyzed the physiological substrate DIMBOAGlc completely. However, E191Q mutant showed very little amount of detectable activity even though the same amounts of proteins were used in the reaction (Fig. 5). The results presented suggest that glutamic acid 191 in maize ß-glucosidase functions as the general acid/base catalyst.

Modifying the carboxyl side chain of the acid/base catalyst at position E191 has effect on pH optimum, as seen in Figure 6. Upon substitution of E191 by Q, the pH optimum value is decreased approximately 1 pH unit (Fig. 6). The E191Q mutation strongly affected the activity dependence on pH; this might be the result of small change in local charge distribution in the active site. The change of pH optimum of E191 mutant confirms that residual activity detected is due to the mutant itself and not to contamination with the wild type.

Activity of the purified proteins was assayed against *p*NPG, *o*NPG, *p*NPGal, *o*NPGal, *p*NPFc and *o*NPFc substrates. Their apparent K_m and k_{cat} values were determined, which are summarized in Table I. As can be seen, the apparent k_{cat} values for the Q mutants were found very low (respectively, 201 and 109-fold lower than the wild

type in *p*NPG and *o*NPG assays). Both wild and mutant enzyme showed the highest activity towards *p*NPFc even though there is still 245-fold activity reduction in the E191Q mutant in comparison to the wild type. It is possible that the substrate *p*NPFc contains methyl group at C5 position of sugar moiety, which makes *p*NP better leaving group. Values of K_m for each enzyme are fairly similar and shifted depending on substrate as seen for *p*NPG and *o*NPG (Table 1). Replacement of Glu with Gln in E191Q mutation may destabilize the transition state and raise the free energy of the transition state.

Interestingly, E191Q mutant showed kinetic enhancement after the addition of 0.4 M Na_2CO_3 that inhibits activity of the wild type enzyme because of elevated pH. One possibility is spontaneous deamination of Q191 at high pH and it could be that the ortho position of NPG is fairly close to the O-linkage of the substrate and may cooperate with the Q residue in order to provide a proton source. The role of the Q is predicted to position the water molecule in hydrolysis as discussed by Burmeister et al. (32) for the myrosinase catalytic mechanism. In contrast, there was not any observed activity enhancement in Q mutant when *o*NPGal was substrate. This could be due to the fact that the K_m value of *o*NPGal for the Q mutant is higher than that of *o*NPGlc.

The structure of active site pocket has been conserved upon Q mutation. The K_m values for the substrates tested are found similar although K_i values for the inhibitor showed slight difference between the Q mutant and the native enzyme. The inhibition constants for the substrates listed in Table 2 indicated that para-nitrophenyl-thio-glucoside inhibits the enzyme competitively. Moving the negatively charged carboxylate group, upon changing the residue from Glu to Asp, results in 0.82 Å increase in distance from the anomeric carbon of the substrate. This must result in less effective stabilization of the transition state from the ground state, therefore cause rate reduction in the glycosylation step due to the change of Glu to Asp.

The replacement of the acid/base catalyst E191 in the active site of maize ß-glucosidase by Q and D by site-directed mutagenesis results in inactivation of the enzyme. Therefore replacement of the acid/base catalyst, E, by the smaller D must change the position and distance of carboxylate group steriochemically and must leave some space in the catalytic center (Fig. 8A, B, C). Thus there was not any substantial activity detected in the D mutant. The only activity detected was with *p*NPG and *o*NPG substrates (10^4x fold reduced in comparison to wild type enzyme). This might be the result of water molecules filling the space created from aspartic acid. It is possible that a water molecule is positioned to O-linkage of substrate and donates a proton to anomeric carbon of activated

sugar resulting in cleavage of the glycosidic linkage. Overall, these data indicate that E191 in maize ß-glucosidases is the acid/base catalyst, and its function in catalysis cannot be performed by an isosteric residue such as glutamine or by a carboxyl group on a shorter side chain such as that of aspartic acid. Therefore, the positional requirements for proton transfer are necessary for ß-glucosidases.