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HEAVY METAL EFFECTS ON β-GLUCOSIDASE ACTIVITY INFLUENCED BY pH AND BUFFER SYSTEMS

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Inhibition of β-glucosidase activity by Cu(II), Zn(II) and Ni(II) was investigated as a function of pH and buffer type. Both factors were found to exert a strong effect on the activity of the enzyme. All three of the investigated heavy metals inhibited the enzyme activity in acetate buffer. At metal concentrations of 0.6 mM, Zn and Ni reduced the enzyme activity by 25–30% under optimal pH conditions (pH 5–5.2). Under the same conditions, Cu showed an even more pronounced inhibitory effect than Zn and Ni. In presence of 0.6 mM Cu, the enzyme activity was lowered by more than 90% in comparison to metal free systems. In contrast to these results, no enzyme inhibition was observed in citrate buffer, even in the presence of 1 mM Cu.

The inhibition of β-glucosidase activity by Cu increased with increasing pH. Inhibition by Zn and Ni was less pH-dependent in the observed pH range (pH 4–5.5). Copper caused a distinct shift in the pH optimum of enzyme activity, whereas this was not the case for Zn or Ni. The effects of buffer and pH on enzyme inhibition by Cu, Zn and Ni were successfully described using a chemical speciation model, based on the assumption that enzyme activity depends on the protonation of the amino acids at the reactive site and that enzyme activity is inhibited by complexation of the reactive sites by the heavy metal cations. The results show the importance of taking chemical conditions and speciation into account when investigating the effect of heavy metal cations on biological systems.

Keywords: β-glucosidase; Heavy metals; Chemical speciation calculations; pH and buffer conditions

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INTRODUCTION

The influence of metal cations on cellulases have been investigated in various studies, as recently reviewed by Goyal and Eveleigh,[1] and it generally has been found that copper and mercury inhibit cellulases. While the experimental data indicate that the sensitivity of cellulases for metals varies among organisms, the variation of experimental conditions makes it difficult, if not impossible, to quantitatively compare results from different studies. Apart from pH differences, in particular, differences in type and concentration of buffers used for the enzyme assays impede such comparisons. Thus, Ng and Zeikus[2] reported different sensitivities of *Trichoderma reseei* and *Clostridium thermocellum* cellulases for trace metals. However, the activity of *Trichoderma reseei* cellulase was measured in 0.1 M citrate buffer, while the activity of *Clostridium thermocellum* cellulase was investigated in 0.2 M sodium acetate buffer. Instead of indicating different metal sensitivities of the two cellulases, the difference may be a consequence of the use of different buffers.

The aim of this work was to investigate the influence of pH and buffer conditions on the interaction between the heavy metals Cu^{2+}, Zn^{2+}, Ni^{2+} and β-glucosidase. Under well-controlled laboratory conditions, β-glucosidase activity was measured in the presence of copper, zinc, and nickel in different buffers and at various pH values. For the interpretation of experimental data, a chemical speciation model was developed, based on the assumption that the activity of an enzyme depends critically on the protonation state of the amino acids at the reactive centre. Additionally, the inactivation of an enzyme may be caused by metals, mainly due to complexation of the amino acids at the catalytic site. However, it also can be caused by metal complexation of amino acids at substrate binding sites or structural sites of the enzyme.

MATERIAL AND METHODS

Activity of β-Glucosidase

The kinetics of the cellobiose cleaving reaction catalysed by β-glucosidase from almonds (EC 3.2.1.21, 5.8 U mg^{-1} (97 nkat mg^{-1}), $M_r \approx 130\,000$, Fluka, Buchs, Switzerland) were studied in test tubes made of glass with 5 ml assays containing 25 mg of cellobiose, 72 nkat (approx. 10^{-6} M) of β-glucosidase and either 0.1 M Na acetate or 0.1 M Na citrate as buffer. The reactions were carried out for 15 min at 25°C. The activities were calculated based on this experimental period. To terminate the reaction, the samples were placed in boiling water for 5 min.

Sample Preparation and HPLC Analysis

Prior to HPLC analysis, samples were desalted by electrodialysis to remove ionic components of the enzyme assay because these interfered with the analysis of sugars. Thereafter, the samples were analysed by a high pressure liquid chromatography system equipped with a refractive index detector as described in Geiger *et al.*[3]

Speciation Calculations

The concentrations of species in solution were calculated using the chemical speciation programme MICROQL as described in Geiger *et al.*[4] Concentrations of the components and stability constants used in the calculations are given in Tables I and II.

TABLE I Data used for speciation calculations in the three scenarios. Model assumptions for the enzyme-proton-metal interactions

	Species	*Model equations*
Fixed component concentrations:	$[HRcentre^-]_{chelate*}$	$[HSuccinate^-] = 10^{5.2}\ [H^+]\ [Succinate^{2-}]$
pH varied between 2 and 6	$[H_2Rcentre]_{chelate}$	$[H_2Succinate] = 10^{9.2}\ [H^+]^2\ [Succinate^{2-}]$
$[H_2CO_3] = 10^{-5}$ M	$[CuRcentre]_{chelate}$	$[CuOxalate] = 10^{6.3}\ [Cu^{2+}]\ [Oxalate^{2-}]$
		$[CuMalonate] = 10^{5.8}\ [Cu^{2+}]\ [Malonate^{2-}]$
	$[NiRcentre]_{chelate}$	$[NiOxalate] = 10^{3.7}\ [Ni^{2+}]\ [Oxalate^{2-}]$
		$[NiMalonate] = 10^{3.24}\ [Ni^{2+}]\ [Malonate^{2-}]$
Total dissolved concentrations (scenarios 1 and 2):	$[ZnRcentre]_{chelate}$	$[ZnOxalate] = 10^{3.88}\ [Zn^{2+}]\ [Oxalate^{2-}]$
		$[ZnMalonate] = 10^{2.96}\ [Zn^{2+}]\ [Malonate^{2-}]$
$[Metal^{2+}] = 6 \cdot 10^{-4}$ M		$[Imid^-]$
$[Acetate^-] = 10^{-1}$ M	$[His^-_{C\text{-}term}]$	$[HImid] = 10^{7.44}\ [H^+]\ [Imid^-]$
$[Rcentre^-] = 10^{-6}$ M	$[HHis_{C\text{-}term}]$	$[CuImid^+] = 10^{4.5}\ [Cu^{2+}]\ [Imid^-]$
	$[CuHis^+_{C\text{-}term}]$	
	$[Gly_{N\text{-}term}]$	[Ethyl]
Total dissolved concentrations (scenario 3):	$[HGly^+_{N\text{-}term}]$	$[HEthyl^+] = 10^{7.8}\ [H^+]\ [Ethyl]$
	$[CuGly^{2+}_{N\text{-}term}]$	$[CuEthyl^{2+}] = 10^{4.7}\ [Cu^{2+}]\ [Ethyl]$
$[Metal^{2+}] = 6 \cdot 10^{-4}$ M	$[HHis^+_{non\text{-}term}]$	$[HImidazole^+] = 10^{7.0}\ [H^+]\ [Imidazole]$
$[Acetate^-] = 10^{-1}$ M	$[CuHis^{2+}_{non\text{-}term}]$	$[CuImidazole^{2+}] = 10^{4.2}\ [Cu^{2+}]\ [Imidazole]$
$[Rcentre^-] = 10^{-6}$ M	$[Asp^-_{non\text{-}term}]$	$[Acetate^-]$ (data in Table 2)
$[His^-_{C\text{-}term}] = 10^{-6}$ M	$[Glu^-_{non\text{-}term}]$	$[Acetate^-]$ (data in Table 2)
$[Gly_{N\text{-}term}] = 10^{-6}$ M		
$[His_{non\text{-}term}] = 4.1 \cdot 10^{-5}$ M		
$[Asp^-_{non\text{-}term}] = 1.4 \cdot 10^{-4}$ M		
$[Glu^-_{non\text{-}term}] = 9.3 \cdot 10^{-5}$ M		

Abbreviations: Rcentre = reactive centre of the enzyme; Imid = 3-(4-imidazolyl)propionic acid; Ethyl = glycylglycine ethyl ester.
*Chelate includes chelate complexes and potential chelate-forming components.

TABLE II Mass action equations used for the calculations. The stability constants[17–20] were adjusted for an ionic strength of 0.1

Experimental values used

$[OH^-] = 10^{-13.78}[H^+]^{-1}$
$[CO_3^{2-}] = 10^{-16.24}[H_2CO_3][H^+]^{-2}$
$[HCO_3^-] = 10^{-6.01}[H_2CO_3][H^+]^{-1}$
$[CuOH^+] = 10^{-7.17}[Cu^{2+}][H^+]^{-1}$
$[Cu(OH)_2] = 10^{-16.41}[Cu^{2+}][H^+]^{-2}$
$[CuHCO_3^+] = 10^{-5.40}[H_2CO_3][H^+]^{-1}[Cu^{2+}]$
$[CuCO_3] = 10^{-9.69}[H_2CO_3][H^+]^{-2}[Cu^{2+}]$
$[HAcetate] = 10^{4.56}[H^+][Acetate^-]$
$[CuAcetate^+] = 10^{1.83}[Cu^{2+}][Acetate^-]$
$[Cu(Acetate)_2] = 10^{3.09}[Cu^{2+}][Acetate^-]^2$
$[Cu(Acetate)_3^-] = 10^{2.66}[Cu^{2+}][Acetate^-]^3$
$[Cu(Acetate)_4^{2-}] = 10^{2.23}[Cu^{2+}][Acetate^-]^4$
$[HCitrate^{2-}] = 10^{5.97}[H^+][Citrate^{3-}]$
$[H_2Citrate^-] = 10^{9.68}[H^+]^2[Citrate^{3-}]$
$[H_3Citrate] = 10^{12.9}[H^+]^3[Citrate^{3-}]$
$[CuCitrate^-] = 10^{5.9}[Cu^{2+}][Citrate^{3-}]$
$[CuHCitrate] = 10^{9.6}[Cu^{2+}][H^+][Citrate^{3-}]$
$[Cu_2Citrate^+] = 10^{8.1}[Cu^{2+}]^2[Citrate^{3-}]$
$[Cu_2(Citrate)_2^{2-}] = 10^{14.8}[Cu^{2+}]^2[Citrate^{3-}]^2$
$[CuCitrateOH^{2-}] = 10^{2.23}[Cu^{2+}][Citrate^{3-}][H^+]^{-1}$
$[ZnOH^+] = 10^{-9.86}[Zn^{2+}][H^+]^{-1}$
$[Zn(OH)_2] = 10^{-18.01}[Zn^{2+}][H^+]^{-2}$
$[ZnHCO_3^+] = 10^{-5.48}[Zn^{2+}][H_2CO_3][H^+]^{-1}$
$[ZnCO_3] = 10^{-12.46}[Zn^{2+}][H_2CO_3][H^+]^{-2}$
$[ZnAcetate^+] = 10^{1.1}[Zn^{2+}][Acetate^-]$
$[Zn(Acetate)_2] = 10^{1.9}[Zn^{2+}][Acetate^-]^2$
$[NiOH^+] = 10^{-10.78}[Ni^{2+}][H^+]^{-1}$
$[Ni(OH)_2] = 10^{-19.21}[Ni^{2+}][H^+]^{-2}$
$[NiHCO_3^+] = 10^{-5.29}[Ni^{2+}][H_2CO_3][H^+]^{-1}$
$[NiCO_3] = 10^{-13.36}[Ni^{2+}][H_2CO_3][H^+]^{-2}$
$[NiAcetate^+] = 10^{0.74}[Ni^{2+}][Acetate^-]$

Estimated values used

$[HRcentre^-]_{chelate} = 10^{5.5}[H^+][Rcentre^{2-}]_{chelate}$
$[H_2Rcentre]_{chelate} = 10^{10.0}[H^+]^2[Rcentre^{2-}]_{chelate}$
$[CuRcentre^+] = 10^{1.83}[Cu^{2+}][Rcentre^-]$
$[CuRcentre]_{chelate} = 10^{5.0-6.0}[Cu^{2-}][Rcentre^{2-}]_{chelate}$
$[HHis^+_{non\text{-}term}] = 10^{7.0}[H]^+[His_{non\text{-}term}]$
$[CuHis^{2+}_{non-term}] = 10^{4.2}[Cu^{2+}][His_{non\text{-}term}]$
$[HGly^+_{N\text{-}term}] = 10^{7.8}[H^+][Gly_{N\text{-}term}]$
$[CuGly^{2+}_{N\text{-}term}] = 10^{4.7}[Cu^{2+}][Gly^-_{N\text{-}term}]$
$[HHis_{C\text{-}term}] = 10^{7.44}[H^+][His^-_{C\text{-}term}]$
$[CuHis^+_{N\text{-}term}] = 10^{4.5}[Cu^{2+}][His^-_{C\text{-}term}]$
$[HAsp_{non\text{-}term}] = 10^{4.56}[H^+][Asp^-_{non\text{-}term}]$
$[CuAsp^+_{non\text{-}term}] = 10^{1.83}[Cu^{2+}][Asp^-_{non\text{-}term}]$
$[HGlu_{non\text{-}term}] = 10^{4.56}[H^+][Glu^-_{non\text{-}term}]$
$[CuGlu^+_{non\text{-}term}] = 10^{1.83}[Cu^{2+}][Glu^-_{non\text{-}term}]$
$[ZnRcentre^+] = 10^{1.1}[Zn^{2+}][Rcentre^-]$
$[ZnRcentre]_{chelate} = 10^{4.0}[Zn^{2+}][Rcentre^{2-}]_{chelate}$
$[NiRcentre^+] = 10^{0.74}[Ni^{2+}][Rcentre^-]$
$[NiRcentre]_{chelate} = 10^{4.0}[Ni^{2+}][Rcentre^{2-}]_{chelate}$

Modelling of Chemical Speciation

The chemical speciation model is based on the assumption that the activity of an enzyme such as β-glucosidase depends critically on the protonation state of the amino acids at the reactive centre of the enzyme, in this case an aspartic acid (Asp) and a glutamic acid (Glu). Since the amino acids of the reactive site of β-glucosidase are not in terminal positions,[5] only the side chain carboxylic groups of Asp and Glu, are able to play an important role in copper binding. The pK values of the two carboxylic acids (4.5 and 5.5) were chosen in order to explain the pH optimum of enzyme activity (see Results and Discussion). They are 0.3–0.5 log units higher than the pK values of a dicarboxylic acid with marginal interactions between the two functional groups such as succinic acid (pK 4.2 and 5.0). This difference may be caused by a lower proton activity within the protein compared to a diluted aqueous solution.

The inactivation of β-glucosidase by metals is thought to be mainly due to complexation of the amino acids at the reactive centre. Since the two amino acids are sterically located near each other, they may not only form binary complexes but also a chelate complex with copper. There are no experimentally determined stability constants available for copper complexation at the reactive site of β-glucosidase. Therefore, we used the stability constant of the simple monocarboxylic acid acetic acid for the binary complexes. For the ternary complex (Asp–Cu–Glu) we considered the structure of a chelate complex with a dicarboxylic acid. From a linear free energy relationship relating the basicity of ligands to complex stability, a stability constant of $\log K$ between 3 and 4 can be estimated. An additional stabilising effect of the chelate formation depends on the steric arrangement of the two functional groups (the length of the chain formed by the chelate). The chelate effect can contribute a $\Delta(\log K)$ of about 4–5 to the overall stability for ideal conditions. This is the case, for example, in the copper–oxalate complex (5-membered chelate ring), whereas the chelate effect is low for longer chains such as in the copper–succinate complex. The involvement of both carboxylates in the enzymatic process of splitting off glucose from cellobiose suggests that the inhibition might be caused by the formation of a chelate complex with copper. On the other hand, the estimated pK values (4.5 and 5.5) of the carboxylic acids indicate rather weak interactions between the two groups, and thus the chelate effect is not expected to be very distinct.

The copper activity effective in enzyme inhibition may be lowered by non-specific binding at any complexing group of the protein. Based on acidity and metal binding constants, the functional groups forming considerable amounts of metal complexes in the experimental pH range (4–5.5) include Glu, Asp, histidine (His) and cysteine (Cys). Their concentration available for copper can only be estimated if the 3-dimensional structure of the enzyme is known. In the case of almond β-glucosidase the information is limited to the amino acid composition. Harder[6] found 139 mol Asp mol^{-1} enzyme (12%), 93 mol Glu mol^{-1} enzyme (8%), 41 mol His mol^{-1} enzyme (4%) and no cysteine. As model compounds to estimate the protonation and copper complex formation constants we chose acetic acid for Glu and Asp, and imidazole for His.

With respect to copper binding, the terminal amino acids have to be treated separately. Isoleucine and glycine are the most probable N-terminal amino acids.[6] An extended cleavage procedure revealed isoleucine and glycine to occupy N-terminal positions in the protein fragments. The stability constants are expected to be similar to those of glycylglycine ethyl ester. C-terminal amino acids in almond β-glucosidase seem to be inaccessible

or modified.[7] In the sense of a conservative estimation, we assumed a His, which has the highest copper complex stability constant, to be in this position. Similar binding properties are represented in the calculations by the model compound 3-(4-imidazolyl)propionic acid.

In order to realise the effects of complex stability at the reactive centre and copper binding to other functional groups of the protein, we modelled three scenarios, namely

(a) "maximal inhibition": only the amino acids of the reactive site react with metals, $\log K_{\text{chelate copper complex}} = 6$,
(b) "intermediate inhibition": like scenario (a), but $\log K_{\text{chelate copper complex}} = 5$,
(c) "minimal inhibition": all reactive groups are available for the metals, $\log K_{\text{chelate copper complex}} = 5$.

RESULTS AND DISCUSSION

Influence of pH on β-Glucosidase Activity

Figure 1 shows the influence of pH on the activity of almond β-glucosidase in 0.1 M Na-acetate and 0.1 M Na-citrate buffer. In both buffers, a maximum activity was found at pH 5. The pH optimum agrees with that

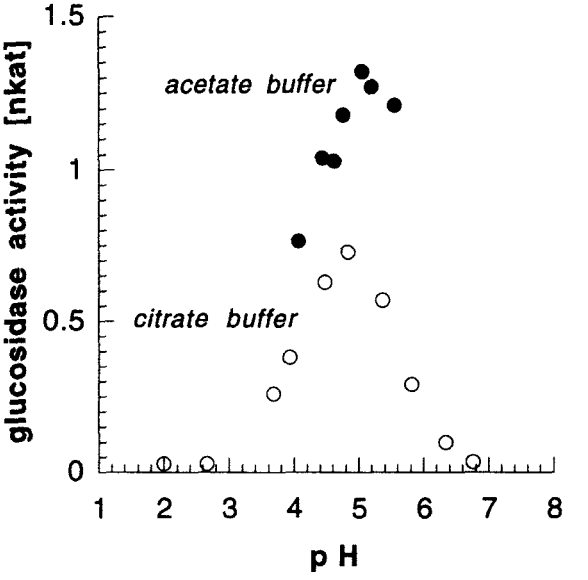

FIGURE 1 Influence of pH on β-glucosidase activity in 0.1 M Na-acetate and 0.1 M Na-citrate buffer at 25°C expressed as rate of glucose production in the first 15 min.

found by Quiquampoix.[8] The β-glucosidase activity in Na-citrate was significantly lower than in Na-acetate. At pH 5, the activity in Na-citrate buffer was 70% of the activity measured in Na-acetate. Becher recommended Na-acetate buffer as the most suitable buffer for activity measurements of almond β-glucosidase and found that assays containing TRIS, phosphate (anionic buffer), glycine (cationic buffer), piperazine or citrate inhibited enzyme activity.[9] Inhibition by citrate was described as competitive while inhibition due to piperazine appeared to be non-competitive. Zaborska and Leszko[10] found that $H_2PO_4^-$ ions are primarily responsible for the inhibition of urease in phosphate buffer. Variation of the ionic strength had only a very weak influence on the catalytic properties of almond β-glucosidase.[9] We found that 25 mM potassium phosphate buffer and a microbial minimal medium mimicking soil solution with the same phosphate concentration showed an identical influence on cellulase activity (data not shown).[4]

The pH optimum can be explained by the speciation of the amino acids of the catalytic site of β-glucosidase. Campbell[11] suggested that the hydrolysis of the glucosidic bound is catalysed only if the two key amino acids, aspartic and glutamic acid, are in the appropriate state: this means that one functional group has to be protonated and the other deprotonated. Deviations from this protonation state would decrease the activity. Figure 2 shows the results of the chemical speciation calculations independent of the choice of buffer. These results indicate that the chosen pK values of the reactive site, 4.5 and 5.5, are appropriate to describe the pH-dependence of the enzyme activity (cf. Figures 1 and 2). However, a comparative quantification of the activity in acetate *vs.* citrate buffers is not possible since the specific interactions between enzyme and buffers are not known.

Influence of Copper on β-Glucosidase Activity in Different Buffers

Figure 3 shows the influence of copper on almond β-glucosidase activity in Na-acetate buffer. The chosen copper concentrations can in fact be found in heavily copper-contaminated soils; Federer and Sticher[12] found 0.3 mM copper in the soil solution of an alkaline soil (pH ≈ 7, total concentration of Cu ≈ 250 mmol kg^{-1} soil). Only at the lowest pH (4.0) and at the lowest copper concentration (0.2 mM) was inhibition not observed. Inhibition increased with the copper concentration. However, the extent of inhibition depended critically on pH. The pH optimum of the enzyme activity shifted towards more acidic conditions with increasing copper concentrations.

Romeu *et al.*[13] found that 1 mM copper affected the almond β-glucosidase activity only slightly. However, comparison with our results is limited

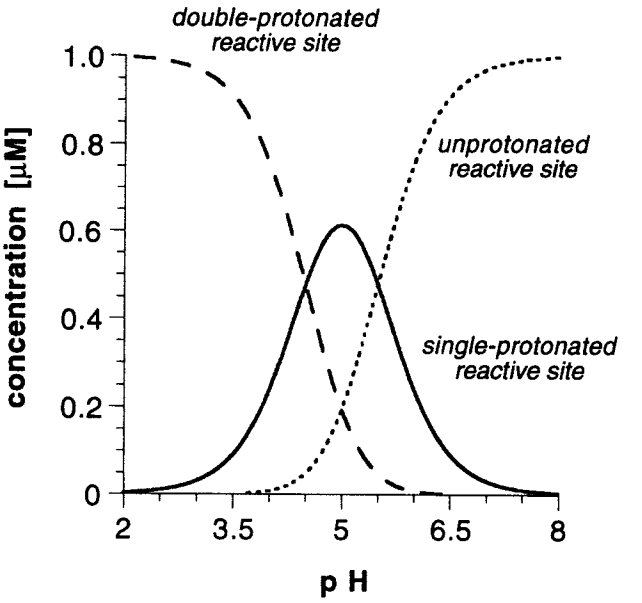

FIGURE 2 Chemical speciation of the reactive site of β-glucosidase (10^{-6} M) in presence of 0.1 M Na-acetate or 0.1 M Na-citrate buffer.

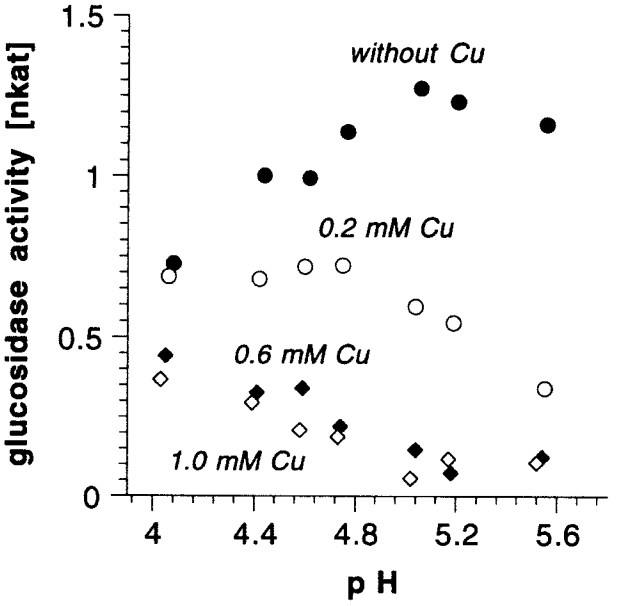

FIGURE 3 Influence of various copper concentrations (0–1 mM) on the activity of β-glucosidase at 25°C in 0.1 M Na-acetate buffer expressed as rate of glucose production in the first 15 min.

because enzyme activity was measured in 50 mM citrate. This ligand complexes copper more strongly than acetate used in this work ($\log K$(Cu-acetate) $= 10^{1.83}$, $\log K$(Cu-citrate) $= 10^{5.90}$, both for $I = 0.1$). The same limitation applies to a comparison with the results of Huang *et al.*[14] They found that 1 mM copper reduced the ferulic acid carboxylase activity of *Pseudomonas fluorescens* to 60% of the original activity. Here, the enzyme assay was performed with 20 mM potassium phosphate buffer at pH 7.3 ($\log K(CuHPO_4) = 10^{2.3}$, $I = 0.15$). The apparent lower sensitivity of ferulic acid carboxylase towards copper may be due to the high amount of copper complexed by phosphate at pH 7.3 or due to lower copper complexing properties of the enzyme.

Iwasaki *et al.*[15] investigated the influence of 1 mM copper on *Trichoderma koningi* cellulases at pH 4 in 0.1 mM acetate buffer and found that the activities of the cellulases were reduced by less than 10% in the presence of copper. This corresponds to results by Geiger *et al.*[3] for *Trichoderma viride* cellulases measured under the same conditions. The comparison of this data with our almond β-glucosidase data suggests that copper inhibits almond β-glucosidase more than *Trichoderma* cellulases.

The speciation of 0.6 mM copper in the presence of 10^{-6} M β-glucosidase in 0.1 M Na-acetate buffer is shown in Figure 4. The calculated amount of

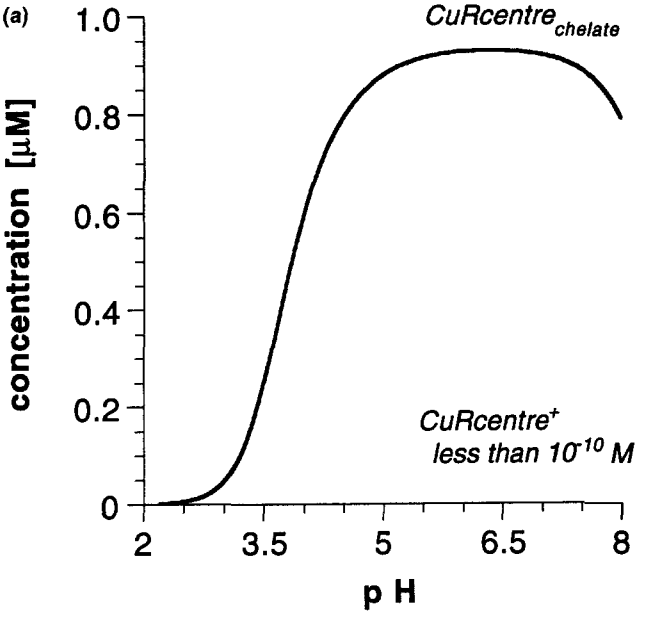

FIGURE 4(a)

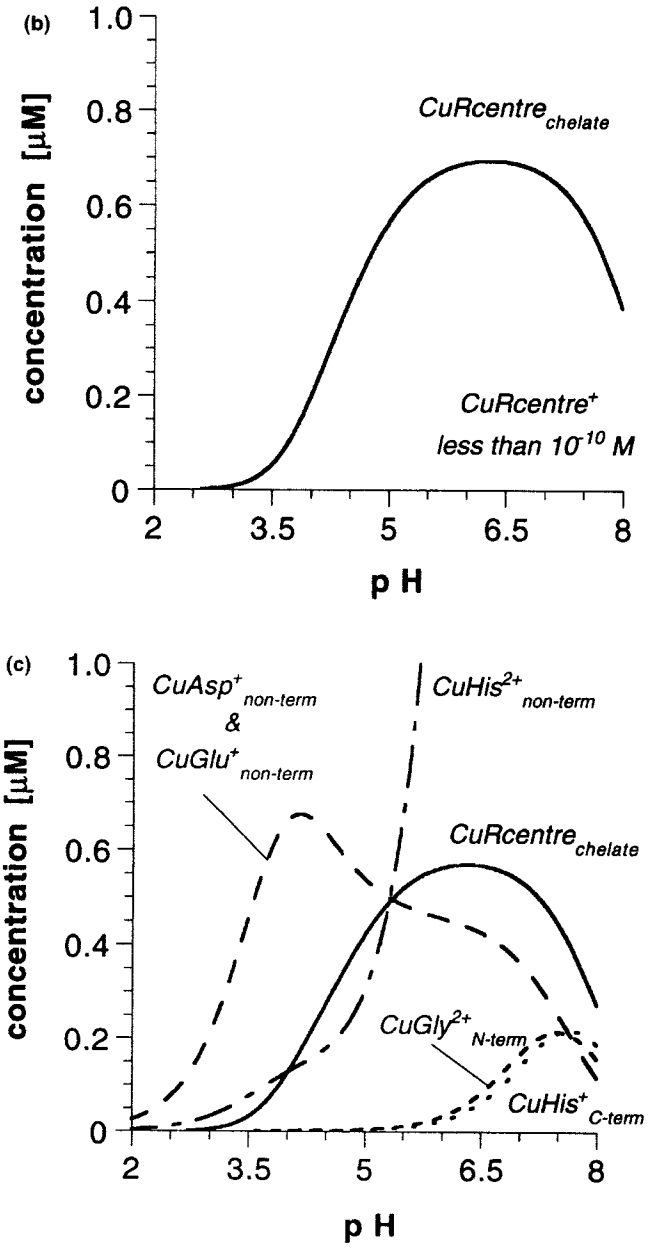

FIGURE 4(b) and (c)

FIGURE 4 Different scenarios of the chemical speciation of β-glucosidase (10^{-6} M) in presence of 0.6 mM copper and 0.1 M Na-acetate buffer. (a) Maximum inhibition: only the reactive centre is taken into account, $\log K(\text{CuRcentre})_{\text{chelate}} = 6$, (b) intermediate inhibition: only the reactive centre is taken into account, $\log K(\text{CuRcentre})_{\text{chelate}} = 5$, (c) minimum inhibition: all reactive groups of the enzyme are taken into account, $\log K(\text{CuRcentre})_{\text{chelate}} = 5$. Only selected species are shown.

copper complexes formed with $\log K = 6$ (scenario 1, Figure 4(a)) corresponds quite well to the measured inhibition at pH 5–5.5 and is slightly higher at lower pH values. Binding of copper to protein functional groups other than the reactive centre does not influence the complexation of the centre due to excess of the metal cation (data not shown). A comparison of scenarios 1 and 2 (Figure 4(a) and (b)) shows the influence of the estimated chelate stability on the copper complex formation at the reactive centre. Although the complex formation as a function of pH corresponds qualitatively to the measured activity, the inhibition cannot be explained quantitatively by copper binding at the reactive site with $\log K = 5$. The underestimation of inhibition is even more distinct if the copper concentration available is lowered due to additional binding sites of the enzyme (cf. scenarios 2 and 3, Figure 4(b) and (c)). On the other hand, we have to consider that additional copper binding could also act in the opposite direction: it may cause a non-specific inhibition, e.g., by unfolding the protein. We conclude that speciation calculations are a useful tool in the qualitative interpretation of enzyme activities. However, for quantitative statements the stability constant of the copper complex at the reactive centre and the knowledge on the effects of copper bound to other protein functional groups are required.

The influence of copper on β-glucosidase in 0.1 M Na-citrate buffer is shown in Figure 5. In this medium, no clear effect of copper on the enzyme activity is obvious in the pH range 2–8. Note that these data do not correspond with those measured in 0.1 M Na-acetate buffer. According to our results, Romeu *et al.*[13] found that 1 mM copper reduced the almond β-glucosidase activity at pH 4.8 in 50 mM citrate buffer by only ≈7%.

Figure 6 shows the copper complexation of amino acids at the reactive site of β-glucosidase in 0.1 M Na-citrate buffer as a function of pH. At pH 2–8 only a very small amount of copper chelate complexes is formed. This explains why copper has no distinct effect on the β-glucosidase activity in Na-citrate buffer, in contrast to Na-acetate buffer solutions (Figure 3).

Comparison of Different Metals

Zinc and nickel inhibited β-glucosidase (Figure 7) activity less than copper at pH 4.5–5.5 (Figure 3). The effect of zinc and nickel depended only slightly on pH in this range and did not cause a significant shift of the pH optimum. According to the chemical speciation calculations (Figure 8), the amount of single-protonated reactive sites is about the same in the case of nickel and zinc and significantly lower in the presence of copper.

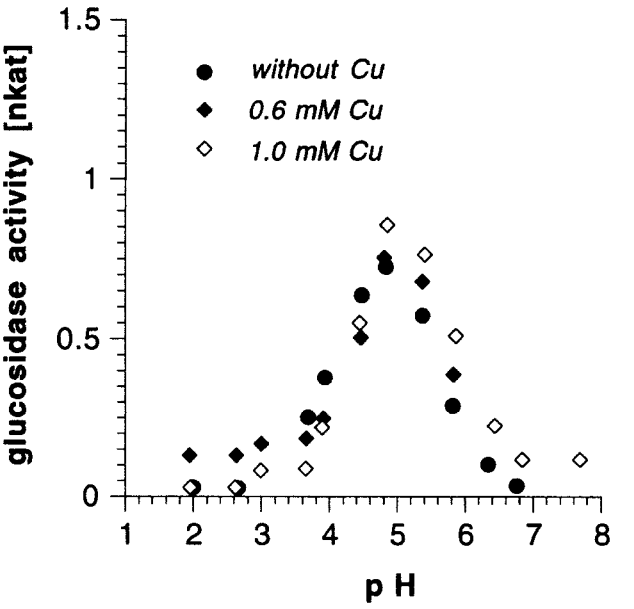

FIGURE 5 Influence of various copper concentrations (0–1 mM) on the activity of β-glucosidase at 25°C in 0.1 M Na-citrate buffer expressed as rate of glucose production in the first 15 min.

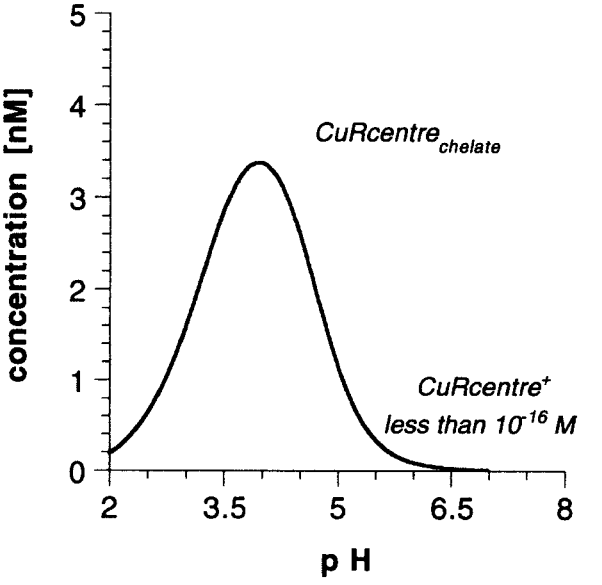

FIGURE 6 Chemical speciation of the reactive site (Rcentre) of β-glucosidase (10^{-6} M) in presence of 0.6 mM copper and 0.1 M Na-citrate buffer. Only selected species are shown.

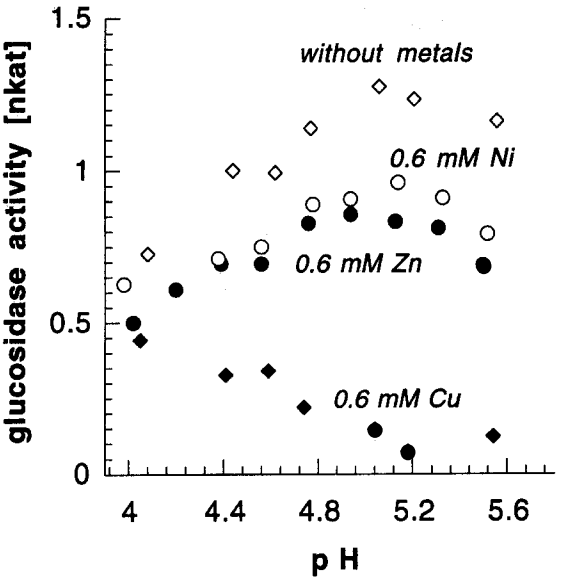

FIGURE 7 Influence of 0.6 mM copper, 0.6 mM nickel and 0.6 mM zinc on the activity of β-glucosidase at 25°C in 0.1 M Na-acetate buffer expressed as rate of glucose production in the first 15 min.

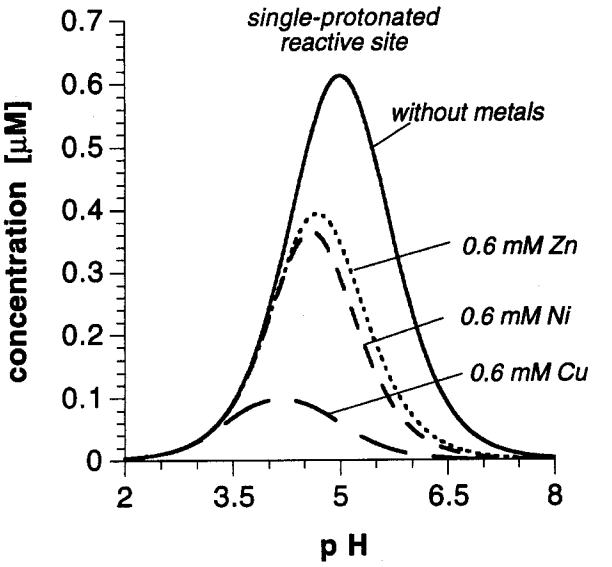

FIGURE 8 Concentration of the single-protonated reactive site (HRcentre) of β-glucosidase (10^{-6} M) in presence of 0.6 mM copper, 0.6 mM nickel or 0.6 mM zinc in 0.1 M Na-acetate buffer.

The chemical speciation model also suggests a significant shift of the pH optimum if copper is added to the system, whereas this is not the case for nickel and zinc. These two observations correspond very well with the experimental data.

CONCLUSIONS

The experimental setup is very important when conducting enzyme assays, especially when investigating the effect of metals on enzyme activity. Using different buffers in enzyme assays may produce non-comparable results. Furthermore, pH is of crucial importance in metal containing systems, since the toxicity of metals towards enzymes may vary strongly with pH depending on the chemical nature of the metal and on the observed pH range.

Sodium citrate and Na acetate, which are often used as buffers in enzyme assays, are found in the environment as by-products of microbial metabolism.[16] Depending on the system that is intended to be investigated, it is important to use the most suitable buffer. It is not only crucial to consider the sensitivity of the enzymes towards the buffers but also the influence of the buffers on the chemical speciation of the investigated metals. Speciation calculations can be used to interpret different sensitivities of an enzyme towards metal ions in different environments, but not to estimate the influence of a buffer towards the enzyme itself.

Our chemical speciation calculations predict that copper inhibits enzyme activity stronger than zinc and nickel. Additionally, the calculations predict that copper, but not zinc and nickel, causes a change in the pH optimum of the enzyme. The calculated data corresponded qualitatively very well with the data measured. Different scenarios suggested that the enzyme inhibition may be quantitatively determined if the stability constant of the metal with the catalytic site is known. Thus, speciation calculations can serve as a useful tool for the interpretation of experimental data on enzyme activity investigated in the presence of metals.

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