FEBS Letters 576 (2004) 46–50 FEBS 28806

Synergetic inhibition of metal ions and genistein on α-glucosidase

Yufang Wang[a,b], Lin Ma[a], Zhong Li[d], Zhiyun Du[a], Zhong Liu[a], Jiangke Qin[a], Xiaodong Wang[b], Zhishu Huang[b], Lianquan Gu[a,b,*], Albert S.C. Chen[c]

[a]School of Chemistry and Chemical Engineering, SUN Yat-sen (Zhongshan) University, 510275 Guangzhou, China
[b]School of Pharmaceutical Science, SUN Yat-sen (Zhongshan) University, 510080 Guangzhou, China
[c]Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong
[d]Shenzhen Hepalink Pharmaceutical Co., Ltd., 518057, Shenzhen, China

Received 18 August 2004; accepted 18 August 2004

Available online 11 September 2004

Edited by Sandro Sonnino

Abstract Inhibition of metal ions and synergetic inhibition of metal ions/genistein on α-glucosidase activity has been investigated. We have examined the inhibitory effect of Cu^{2+}, Ni^{2+}, Mg^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+}, Ca^{2+}, Pb^{2+}, Ag^{+}, V^{5+}, V^{4+} and Mn^{2+} ions. The results show that the nature of the inhibition was reversible, slow-binding, non-competitive, and the K_i values of some ions such as Cu^{2+}, Ni^{2+} and Zn^{2+} range from 10^{-5} to 10^{-6} M. Moreover, synergetic inhibitory effect of metal ions and genistein on α-glucosidase were studied with kinetics method. It is concluded that the inhibitory effect was much greater when both of them were added to the reactant solution simultaneously than that they were added, respectively, which suggests that the inhibitors seem to bind to the different sites of α-glucosidase at the same time. Furthermore, the mechanism of the synergetic inhibition was examined by spectrophotometry.

Keywords: Synergetic inhibition; α-Glucosidase; Metal ions; Genistein

1. Introduction

Glycosidases(glycohydralase) are ubiquitous in the living world and are involved in carbohydrate metabolism. They catalyze the cleavage of glycosidic linkage in glycosides, oligosaccharides and polysaccharides as well as such complex carbohydrate as those present in glycolipids and glycoproteins [1]. Glycosidase is not only essential to carbohydrate digestion, but also vital for the processing of glycoproteins and glycolipids. Glycosidases are also involved in a variety of metabolic disorders and other diseases such as diabetes, viral attachment and cancer formation. Because of their importance, glycosidase inhibitors can be as a vital tools for studying their mechanisms of action and act as therapeutic agents for some degenerative diseases [2–5].

Systematic comparison of the over 2000 known primary amino acid sequences of glycosyl hydrolases led to the group classification of 76 families. α-Glucosidases are located in the brush-border surface membrane of intestinal cells [6], and are the key enzymes of carbohydrate digestion [7]. It specifically hydrolyzes the α-glucopyranosidic bond, thereby releasing an α-D-glucose from the non-reducing end of the sugar. α-Glucosidase has been found to contribute to the glycosylation of human immunodeficiency virus type I (HIV-I) 120 and inhibitors of α-glucosidase can block the viral infection [8–10]. Recently there has been widespread interest in these enzymes, partly because of their potential as therapeutic targets.

The inhibition of enzyme by metal ions is of considerable importance and has been studied extensively. Metal ions such as copper and zinc ions are physiologically important ions, and play a crucial role in many biological functions. It was reported that some metal ions such as zinc, nickel, vanadium and so on were helpful for the therapy of diabetes [11]. However, the inhibition of α-glucosidase by metal ions has never been reported, and the nature of the inhibition of α-glucosidase activity by them is not yet understood.

In this paper, we reported, for the first time, the inhibition of metal ions on α-glucosidase, and the results of our detailed investigations of the interaction of various inhibitors with the enzyme. Since the physiological substrate for α-glucosidase is a disaccharide (maltose), there must be at least two sugar binding sites at the enzymatic active site. The study in the simultaneous presence of two or more inhibitors was undertaken to examine the specificity of these binding sites and their topography. We found the synergetic interaction of metal ions and some natural products such as genistein on α-glucosidase.

The results reported here provided an insight into how metal ions influence the enzyme activity, and how the synergetic effect modulates the individual inhibition of each component to prevent the substrate binding to the enzyme.

2. Materials and methods

2.1. Reagents

p-Nitrophenyl (PNP) glycosides and α-glucosidase (from baker's yeast) used in this study were both purchased from Sigma (St. Louis, MO, USA).

Genistein was isolated and purified from soybean by our laboratory.

Analytic grade metal salts were purchased from Sigma. In all the experiments deionized water and phosphate buffers are used.

2.2. Enzyme assays

The inhibitory effect of various metal ions on α-glucosidase was done as described previously [12,13]. In brief, α-glucosidase activity was assayed using 50 mM phosphate buffer at various pH (6.8 or 7.0), and the appropriate PNP glycosides (at 1 mM) were used as substrates. The concentration of the enzymes is specified in each experiment. Metal

* Corresponding author. Fax: +86-20-84110272.
E-mail addresses: cedc42@zsu.edu.cn, wanying5@yahoo.com (L. Gu).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.08.059

ions at the designated concentration was added to the enzyme solution and incubated at 37 °C for 0.5 h, and the substrate was then added to initiate the enzyme reaction. The enzyme reaction was carried out at 37 °C for 30 min. Product (PNP) was monitored spectrophotometrically by measuring the absorbance ($\lambda = 400$ nm). One unit of α-glucosidase is defined as the amount of enzyme liberating 1.0 μmol of PNP per minute under the assay conditions specified.

2.3. Dialysis experiment

α-Glucosidase (0.5 ml, 10 U/ml) and metal (10 μM) were mixed for appropriate times at 30 °C were dialyzed against phosphate buffer (5 mM, pH 6.8) at 4 °C for 24 h, changing the buffer every 12 h. Another 0.5 ml aliquot was kept at 4 °C for 24 h without dialysis. The residual enzyme activity in both dialysis tubes and aliquot without dialysis were determined as described in Section 2.2.

2.4. Kinetics of enzyme inhibition

The enzyme reaction was performed in the above reaction conditions with inhibitors of various concentrations. Inhibition types for the inhibitors were determined by Dixon plot and its replot of slope versus the reciprocal of the substrate concentration.

2.5. Circular dichroism (CD) assay

The characterization of secondary structure of α-glucosidase in the buffer solution with or without inhibitors was examined with circular dichroism micrometry. The data obtained from the experiments were dealt with the professional software *Secondary Structure Estimation* and *Origin* 6.0.

3. Results and discussion

Metal ions such as copper and zinc ions are physiologically important ions, and play a crucial role in many biological functions. However, the inhibition of α-glucosidase by metal ions has never been reported, and the mechanism of the inhibition of α-glucosidase activity by them is not yet understood. In the course of development of α-glucosidase inhibitors from natural sources, we found metal ions as candidates for α-glucosidase inhibitors, we first examined which metal ions specifically inhibit α-glucosidase.

In order to establish the effect of metal ions on the α-glucosidase, we have tested the influence of different chloride salts of these metals on the activity of the α-glucosidase (Fig. 1). The various metals of 1 mM were added to reaction solution of α-glucosidase and PNP-α-glucopyranoside, and the formation of *p*-nitrophenyl was monitored spectrophotometrically by measuring the absorbance ($\lambda = 400$ nm).

Transitions state metals such as copper, vanadium, nickel and zinc showed an almost complete inhibition, whereas iron and boron gave a mediated inhibition. However, magnesium gave a weak inhibition and manganese, calcium, aluminum were without effect (Table 1).

The activity of α-glucosidase is inhibited by metal ions in a dose–responsive manner (Figs. 2–4). We therefore performed dose–response experiments for further investigating the inhibitory effect of copper, nickel and zinc on α-glucosidase, and determined the IC_{50} values (Figs. 2–4). Enzyme was strongly inhibited by Cu^{2+}, with an IC_{50} value of 2.28 μM (Fig. 2) and an IC_{50} value of 23.3 of Ni^{2+} (Fig. 4), as well as of 99.3 μM of Zn^{2+} (Fig. 3). It is showed that the activity of α-glucosidase is weakened by metal ions in a dose–responsive manner. This suggests that the metal ions have strong affinity towards α-glucosidase.

The inhibition mode of the metals was examined. Non-competitive inhibition was partially observed because the K_i values varied with the length of pretreated time of α-glucosidase with metal ions. When copper ions and substrate was added simultaneously, showing a K_i value of 500 nM (data not shown), whereas, when the enzyme was pretreated with copper ions (2.22 μM) for 1 h, the K_i value was 10 μM. Double-reciprocal plots of α-glucosidase with copper revealed that the nature of enzyme inhibition was non-competitive (shown in Fig. 5). At this point, the K_i value was calculated using the values of V_{max} obtained at 2 and 2.9 μM of copper. The results showed that the inhibition of metal ions on α-glucosidase seems to be a slow-binding non-competitive mode.

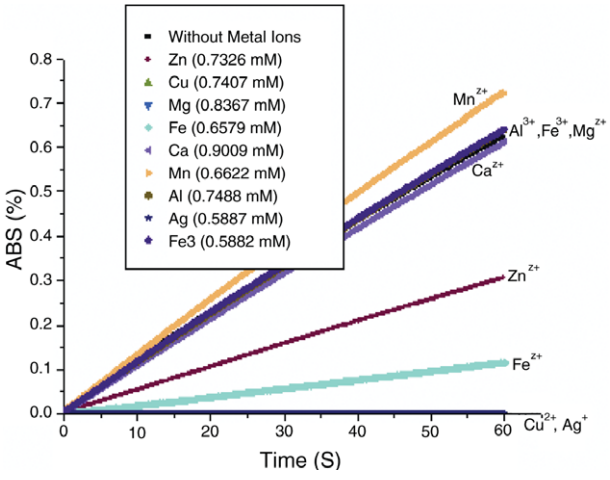


Fig. 1. Inhibition by various metal ions against α-glucosidase. (Enzyme solutions were treated with designated concentrations of metal ions.)

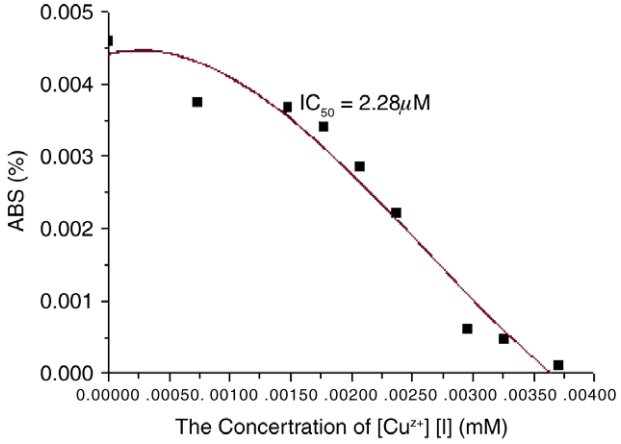


Fig. 2. Inhibition by $CuCl_2$ against α-glucosidase.

Table 1
Inhibition of α-glucosidase by various metal ions

Metal ions	Cu^{2+}	Zn^{2+}	V^{4+}	Ni^{2+}	B^{3+}	Fe^{2+}	Mn^{2+}, $Mg^{2+}Ca^{2+}Ag^{2+}$, Al^{3+}, Fe^{3+}
IC_{50} (μM)	2.28	99.3	44.8	23.3	2291	217	–

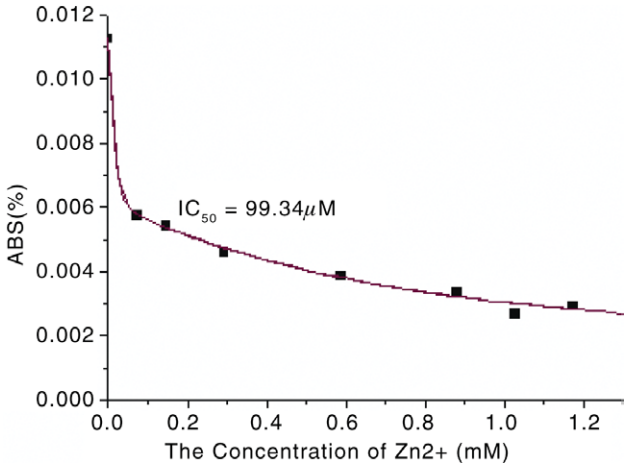


Fig. 3. Inhibition by $ZnCl_2$ against α-glucosidase.

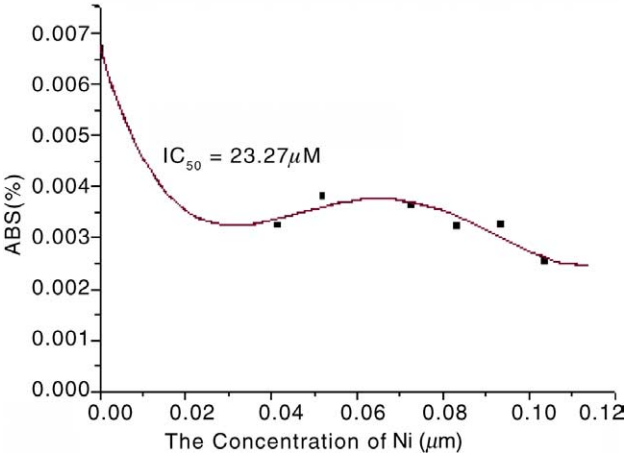


Fig. 4. Inhibition by $NiCl_2$ against α-glucosidase.

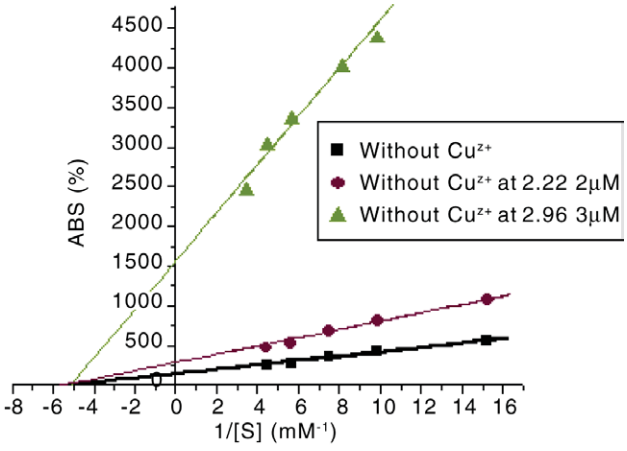


Fig. 5. Double-reciprocal plots of the inhibition kinetics of yeast α-glucosidase by copper. α-Glucosidase (50 μl, 10 U/ml) that was treated first with copper ions for 1 h at 37 °C, then treated with designed concentration of PNP–α-glucopyranoside to initiate the enzyme reaction.

Because the percent of α-helix structure relate to the hydrophobic property of the protein molecular, and the higher content of α-helix within the second structure of the protein, the more stronger hydrophobic property the molecular have. However, two subunits of α-glucosidase [14] must form somewhat hydrophobic domain (active center) to hydrolyze the substrate, poor hydrophobic surroundings led to the failure of the formation of active center.

In order to study the mechanism of the inhibition of metal ions, the influence of inhibitors on the secondary structure of α-glucosidase was examined with circular micrometry. The results in Table 2 showed the decline in the percent of α-helix within the secondary structure of α-glucosidase when the enzyme inhibited by metal ions alone. The results obtained from CD assay showed that the metal ions could increase the hydrophilic of α-glucosidase and weaken the hydrophobic property (see Table 2). Therefore, the activity of enzyme was influenced and inhibited. In present study, we can concluded that metal ions offer a possibility of being developed as successful α-glucosidase inhibitor.

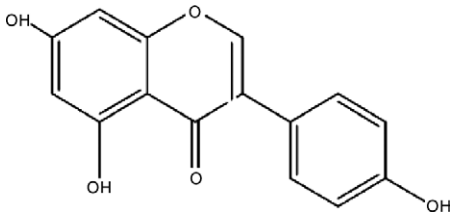
Since there could be more than one active site on the molecular of α-glucosidase, we combined other natural products with known inhibitory effects against the enzyme. In our experiments, we chose genistein (from soybean), at both high and low concentrations as the counterpart inhibitor against α-glucosidase in the experiments demonstrating synergetic inhibition.

Genistein (5,7,4′-trihydroxy-isoflavone, as shown in Scheme 1) belongs to the isoflavonoid family and is the isoflavone of greatest interest in soy protein. Most of the studies have focused on the pharmacological activities of genistein as a tyrosine kinase inhibitor, and its chemoprotectant activities against cancers and cardiovascular disease, as well as its phytoestrogen activity. Recently, It has been reported that genistein could be a potent α-glucosidase inhibitor [2,15].

In our study, the inhibition tests were performed in solutions containing the metal ions alone or in mixture with genistein. As shown in Figs. 6–8, though, genistein, and Copper ion

Table 2
The influence of metal ions on the second structure of α-glucosidase

Metal ions	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
Without inhibitor	37.8	16.4	17.0	28.8
Cu^{2+} (1.33 μM)	25.0	16.6	22.7	35.7
Gen (18.3 μM)	5.1	0.0	38.6	56.4
Zn^{2+} (189 μM)	27.4	6.8	27.2	38.7
V^{4+} (133 μM)	20.9	19.9	21.5	37.7
Ni^{2+} (52 μM)	25.1	10.7	26.2	37.9
Mg^{2+} (768 μM)	33.3	17.8	18.6	30.2



Scheme 1. The structure of genistein (5,7,4′-trihydroxyisoflavone).

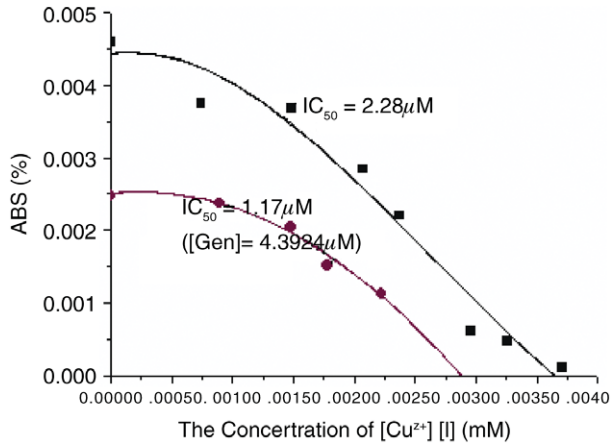


Fig. 6. The synergic inhibition of Cu^{2+} and genistein on α-glucosidase.

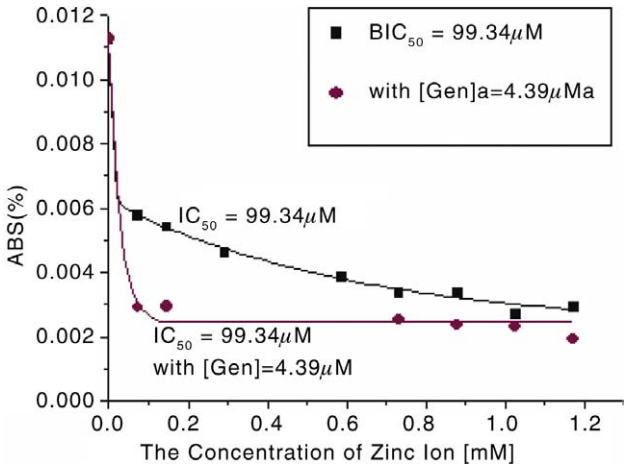


Fig. 7. The synergic inhibition of Zn^{2+} and genistein on α-glucosidase.

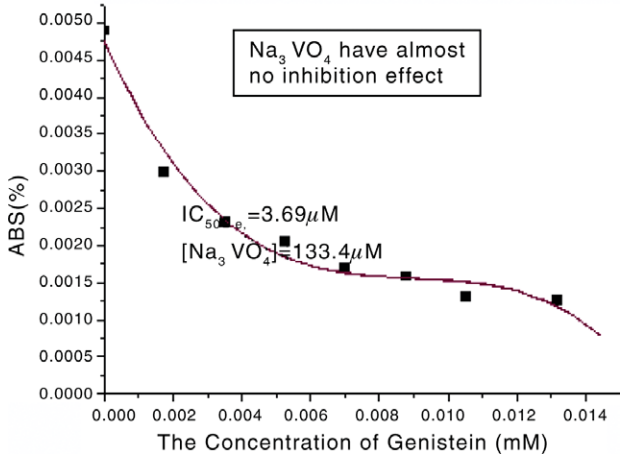


Fig. 8. The synergic inhibition of V^{5+} and genistein on α-glucosidase.

alone at concentrations (10.4 and 2.28 μM, respectively) were demonstrated for inhibiting α-glucosidase by 50%, but when combined in pairs, the IC_{50} value of the Cu^{2+}/genistein, were decreased to 4.39 and 1.1 μM, respectively. Same phenomenon was observed as concerned as Zn^{2+}/genistein, which also showed significant synergy at the test concentration. The IC_{50} value of Zn^{2+} declined from 99.3 to 30 μM with genistein of 4.39 μM.

On the other hand, two others, V/genistein also showed synergetic inhibition at the test concentration, although V^{5+} had almost little inhibitory activity alone on α-glucosidase. The IC_{50} value of genistein declined from 10.4 to 3.69 μM with V^{5+} of 0.133 mM.

Fig. 6 shows that both genistein and copper ions have inhibitory effect and that the mixture of the two compounds results in an additive effect of their inhibition activities. Indeed, the plots corresponding to the results obtained on mixtures are higher than those calculated by summing the effects of each compound alone. Zinc ions and genistein showed the same additive pattern. As shown in Figs. 6 and 7, though, genistein, at low concentrations when combined with metal ions in pairs, the inhibition activity of the M^{2+}/genistein, were remarkably high.

In order to study the mechanism of the synergetic inhibition of metal ions and genistein, the influence of both inhibitors on the secondary structure of α-glucosidase was examined with circular micrometry. The results in Table 3 showed the decline in the percent of α-helix within the secondary structure of α-glucosidase when the enzyme inhibited by the mixtures of genistein and metal ions.

The mechanism of the synergetic effect observed remains unknown. It is possible that the compounds bound to the different sites of the enzyme with non-covalent bond, thus influenced the hydrophilic and hydrophobic property of the molecular of α-glucosidase. The data shown in Table 3 could demonstrate above hypothesis. The percent of α-helix within the secondary structure of α-glucosidase declined to such a extend when the enzyme inhibited by the mixtures of genistein and metal ions, that the hydrophilic property of α-glucosidase increased much. Moreover, the mixtures could increase the hydrophilic and weaken the hydrophobic (see Table 3) properties more than the summing effects of each compound alone. Which led to the strong inhibitory effect on α-glucosidase since poor hydrophobic surroundings led to the failure of the formation of active center, therefore, the inhibitory effect of the combination of genistein metal ions was stronger than that of each components alone.

Our results are very similar to those found in previous studied [15], which showed that there existed synergetic inhibitory effect when genistein and D-glucose, a very poor

Table 3
The synergic inhibition of M^{2+} and genistein (18.3 μM) on α-glucosidase

Metal ions	Conc. (μM)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
Without inhibitor		17.9	17.5	25.2	39.3
Genistein	18.3	11.4	0.0	33.2	55.5
(Cu/Gen)	1.33	10.2	0.0	37.7	52.1
(Zn/Gen)	189	10.3	0.0	36.0	53.7
(V/Gen)	13.3	10.8	0.0	24.1	65.1
(Ni/Gen)	52	7.1	0.0	35.4	57.5
(Mn/Gen)	117.7	4.3	0.0	34.2	61.5
(Mg/Gen)	15.4	13.1	0.0	34.5	52.4

competitive inhibitor of α-glucosidase, added to the reactants solution at the same time. However, our results are different on the question of the interaction of metal ions with genistein, since the metal ions are non-competitive inhibitors. The results show that interaction also existed among non-competitive inhibitors, which suggested there were at least more than one binding site other than active center where the inhibitors could bind.

What is reported here provided an insight into how metal ions influence the enzyme activity, and how the synergetic effect modulates the individual inhibition of each component to prevent the substrate binding to the enzyme. Similar trend has been also observed regarding the inhibition and the synergetic effect of metal ions on yeast α-glucosidase at various pH values (6.8–7.2).

The synergy activity may also be important in human health because oral administration of specific α-glucosidase inhibitor could effectively improve hyperglycemia as well as diabetic complications [16,17]. So our future research should be focused on understanding the mechanism of the synergetic inhibition against α-glucosidase located in the brush-border surface membrane of intestinal cells, since whose optimal pH value is different from that of yeast α-glucosidase, and designing novel drugs derived from combined inhibitors of α-glucosidase beneficial to human health.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (Grant No. 20032021). The authors thank Dr. Linkun An, Dr. Xuepu Mao and Ms. Bin Huang for their helpful assistance.

References

[1] Yao, X. (2001) A Dissertation for the Degree of Doctor of Philosophy, 2001 UMI ProQuest, DAI–B 61/12, June, p. 6464.
[2] Lee, D. and Lee, S. (2001) FEBS Lett. 501, 84–86.
[3] Gruters, R.A., Neefjes, J.J., Tersmette, M., De Goede, R.E.Y., Tulp, A., Huisman, H.G., Miedema, F. and Ploegh, H.L. (1987) Nature 330, 74–77.
[4] Dennis, J.W., Laferte, S., Waghorne, C., Breitman, M.L. and Kerbel, R.S. (1987) Science 236, 582–585.
[5] Winchester, B. and Fleet, G.W. (1992) Glycobiology 2, 199–210.
[6] Hirsh, A.J., Yao, S.Y., Young, J.D. and Cheeseman, C.I. (1997) Gastroenterology 113, 205–211.
[7] Caspary, W.F. (1978) Lancet 1, 1231–1233.
[8] Fischer, P.B., Karlsson, G.B., Butters, T.D., Dwek, R.A. and Platt, F.M. (1996) J. Virol. 70, 7143–7152.
[9] Fischer, P.B., Karlsson, G.B., Dwek, R.A. and Platt, F.M. (1996) J. Virol. 70, 7153–7160.
[10] Walker, B.D., Kowalski, M., Goh, W.C., Kozarsky, K., Krieger, M., Rosen, C., Rohrschneider, L., Haseltine, W.A. and Sodroski, J. (1987) Proc. Natl. Acad. Sci. USA 84, 8120–8124.
[11] Tsujii, E., Muroi, M., Shiragami, N. and Takatsuki, A. (1996) Biochem. Biophys. Res. Commun. 220, 459–466.
[12] Muroi, M., Ando, O., Bols, M. and Takatsuki, A. (2000) Biosci. Biotechnol. Biochem. 64, 1103–1105.
[13] Segel, I.H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme System. Wiley, New York. pp. 100–202.
[14] Loge, J., Maier, T., Liebl, W., Hoffmann, V. and Sträter, N. (2003) J. Biol. Chem. 278, 19151–19158.
[15] Wang, Y., Ma, L., Pang, C., Huang, M., Huang, Z. and Gu, L. (2004) Bioorg. Med. Chem. Lett. 14, 2947–2950.
[16] Koyama, M., Wada, R., Mizukami, H., Sakuraba, H., Odaka, H., Ikeda, H. and Yagihashi, S. (2000) Metabolism 49, 347–352.
[17] Rosenstock, J. and Raskin, P. (1988) Cardiol. Clin. 6, 547–560.