

## RESEARCH ARTICLE

***N*-glycosylation improves the pepsin resistance of HAP phytases by enhancing the stability at acidic pH and reducing the pepsin accessibility to peptic cleavage sites**

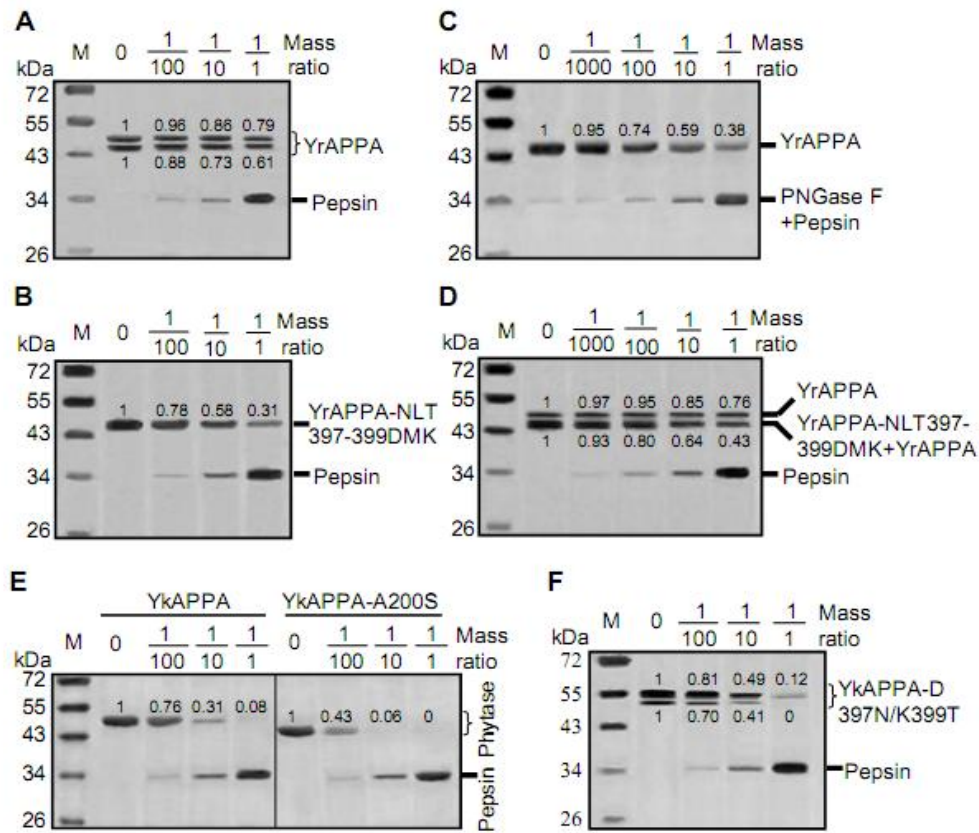
Canfang Niu, Huiying Luo, Pengjun Shi, Huoqing Huang, Yaru Wang, Peilong Yang,  
Bin Yao<sup>1</sup>

*Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research  
Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's  
Republic of China*

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<sup>1</sup>Corresponding author. Address: Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, P. R. China. Tel.: +86 10 82106053; fax: +86 10 82106054.

*E-mail addresses:* binyao@caas.cn; yaobin@caas.cn (B. Yao).



**Supplementary FIG. S1.** SDS-PAGE analysis of the proteolytic products of the wild type and mutant *Yersinia* phytases produced in *P. pastoris* after pepsin treatment at the pepsin/phytase mass ratios of 1/100 to 1/1, pH 2.0 and 37°C for 2 h. (A) YrAPPA. (B) YrAPPA-N397D/L398M/T399K. (C) YrAPPA with glycosylation removal with PNGase F. (D) Enzyme mixture of YrAPPA and YrAPPA-N397D/L398M/T399K. (E) YkAPPA and YkAPPA-S200A. (F) YkAPPA-D397N/K399T. The protein band was quantified using the Image J software.

**Supplementary Table S1** PCR primers for wild-type YeAPPA, YkAPPA and YrAPPA and their mutants

Enzymes <sup>a</sup>	Templates	Primers <sup>c</sup>
YeAPPA (wild-type)	pEASY-T3 -YeAPPA <sup>b</sup>	Forward: 5'-CGCGAATTCGCCCGATTGCTACACCGCC-3' Reverse: 5'-GATGCGGCCGCTTAAATATGGCAGGCTGGCTCGA-3'
<b>YeAPPA-A200S</b>	pEASY-T3 -YeAPPA	Forward: 5'-TGAATTTTICGGCATCGCCTTATTGCAAG-3' Reverse: 5'-CTTGCAATAAGGCGATGCCGAAAAATTCA-3'
<b>YeAPPA-D397N/M398L/K399T</b>	pEASY-T3 -YeAPPA	Forward: 5'-TGCCGAGAAATTAACCTGACAAACAAC-3' Reverse: 5'-GTTGTTTTCAGGTTTAAATTTCTCGGCA-3'
YkAPPA (wild-type)	pEASY-T3 -YkAPPA <sup>b</sup>	Forward: 5'-CGCGAATTCGCACCGCTTGCAGCACAATCTAC-3' Reverse: 5'-GATGCGGCCGCTTAAATATGGCAGGCTGGCTCG-3'
<b>YkAPPA-S200A</b>	pEASY-T3 -YkAPPA	Forward: 5'-TGCTGAATTTTCAGCCTCACCTTATTG-3' Reverse: 5'-CAATAAGGTGAGGCTGCAAAATTCAGCA-3'
<b>YkAPPA-D397N/K399T</b>	pEASY-T3 -YkAPPA	Forward: 5'-GATAAGTTAAACTTGAACAACAACCCGG-3' Reverse: 5'-CCGGGTTGTTTGTCAAGTTTAACTTATC-3'
<b>YkAPPA-L197V</b>	pEASY-T3 -YkAPPA	Forward: 5'-AGGTG@TGAATTTTCGGCCTCACCTTA-3' Reverse: 5'-TAAGGTGAGGCCGAAAAATTCACCACCT-3'
YrAPPA (wild-type)	pEASY-T3 -YrAPPA <sup>b</sup>	Forward: 5'-CTTGAATTCGCCCGGTGATAACCGCACC-3' Reverse: 5'-TAGCGGCCGCTTAAATATGGCAGGCTGGCTC-3'
<b>YrAPPA-N397D/L398M/T399K</b>	pEASY-T3 -YrAPPA	Forward: 5'-GAGAAATTAGATATGAAACTAATCCTG-3' Reverse: 5'-CAGGATTAGTTTTCATATCTAATTTCTC-3'
<b>YrAPPA-L396V</b>	pEASY-T3 -YrAPPA	Forward: 5'-GAATGTTGAGAAAGTAAACCTGACAAC-3' Reverse: 5'-AGTTGTCAGTTTATCTTCTCAACATTC-3'

<sup>a</sup> The introduced mutations in this study are bold.

<sup>b</sup> These genes and enzymes are the products of our previous studies (D. Fu, Z. Li, H. Huang, T. Yuan, P. Shi, H. Luo, K. Meng, P. Yang, B. Yao, Appl Microbiol Biotechnol 90:1295–1302, 2011, doi: 10.1007/s00253-011-3171-0; D. Fu, H. Huang, H. Luo, Y. Wang, P. Yang, K. Meng, Y. Bai, N. Wu, B. Yao, Enzyme Microb Tech 42: 499–505, 2008, doi:10.1016/j.enzmictec.2008.01.014; H. Huang, H. Luo, Y. Wang, D. Fu, N. Shao, G. Wang, P. Yang, B. Yao, Microbiol Biotechnol 80:417–426, 2008, doi: 10.1007/s00253-008-1556-5).

<sup>c</sup> All mutation sites in primers are underlined and the restriction enzyme sites incorporated into the primers are italic.

**Supplementary Table S2** The *N*-glycosylation levels and enhanced pepsin resistance of *N*-glycosylated phytases

Mass ratio of pepsin/phytase	Enhanced pepsin resistance by <i>N</i> -glycosylation of phytases (fold) <sup>a</sup>				
	YeAPPA-DMK397-399NLT	YeAPPA-A200S	YkAPPA	YkAPPA-D397N/K399T	YrAPPA
1/1000	52.2	44.9	0.3	0.4	0.0
1/500	50.6	44.7	0.5	0.8	0.1
1/200	--	--	0.7	1.3	0.2
1/100	--	--	1.3	2.5	0.3
1/40	--	--	9.0	17.5	0.4
1/20	--	--	--	--	0.5
1/10	--	--	--	--	0.6
1/1	--	--	--	--	1.5
Glycosylation level (%)	1.1-1.5	11.5	11.5	13.21-16.36	1.1-11.5
<i>N</i> -glycosylation motifs	NLT	NFS	NFS	NFS+NLT	NLT

--" indicates that the phytase lost all activity after pepsin treatment for 2 h.

a "fold" value was calculated as the ratio of the increased residual activity by *N*-glycosylation of phytase after pepsin treatment to the residual activity of pepsin-treated *N*-deglycosylated or nonglycosylation counterparts. The residual activity was expressed as percentage of activity of untreated enzyme. The *N*-glycosylation level of each phytase was calculated by the proportion of the *N*-glycans accounting for the molecular masses.