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The consensus concept for thermostability engineering of proteins: further proof of concept

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Previously, we calculated a consensus amino acid sequence from 13 homologous fungal phytases. A synthetic gene was constructed and recombinantly expressed. Surprisingly, consensus phytase-1 was 15–26°C more thermostable than all parent phytases used in its design [Lehmann *et al.* (2000) *Protein Eng*., 13, 49–57]. In the present study, inclusion of six further phytase sequences in the amino acid sequence alignment resulted in the replacement of 38 amino acid residues in either one or both of the new consensus phytases-10 and -11. Since consensus phytase-10, again, was 7.4°C more thermostable than consensus phytase-1, the thermostability effects of most of the 38 amino acid substitutions were tested by site-directed mutagenesis. Both stabilizing and destabilizing mutations were identified, but all affected the stability of the enzyme by <3°C. The combination of all stabilizing amino acid exchanges in a multiple mutant of consensus phytase-1 increased the unfolding temperature from 78.0 to 88.5°C. Likewise, back-mutation of four destabilizing amino acids and introduction of an additional stabilizing amino acid in consensus phytase-10 further increased the unfolding temperature from 85.4 to 90.4°C. The thermostabilization achieved is the result of a combination of slight improvements from multiple amino acid exchanges rather than being the effect of a single or of just a few dominating mutations that have been introduced by chance. The present findings support the general validity of the consensus concept for thermostability engineering of proteins.

Keywords: consensus sequence/phytase/protein engineering/thermostabilization

Introduction

The (re-) design of better or even novel molecules is the overall goal of protein engineering (Forrer *et al.*, 1999). In recent years, the emergence of ‘directed evolution’ approaches (Steipe, 1999; Petrounia and Arnold, 2000; Arnold, 2001; Wintrode and Arnold, 2001) has strongly facilitated the targeted improvement of enzymes. Directed evolution has been used for improving the thermo- and solvent stability, the catalytic properties, and even the expression rate of proteins; however, directed evolution strongly depends on a highly discriminating screening assay or selection protocol, which may not always be available or may not even be feasible.

Besides the rather successful directed evolution approaches, stabilizing amino acid substitutions have been predicted successfully using various rational concepts and/or by comparison with the sequence or 3D-structure of a homologous protein from a thermophilic organism. The success rate in most of these cases has been rather low. One of the most prominent exceptions has been reported by van den Burg *et al.* (van den Burg *et al.*, 1998) who applied different rational and semi-rational approaches to the stabilization of a thermolysin-like protease. They came up with a mutant showing a 21°C increase in thermostability which allowed the protease to be active at 100°C. Furthermore, a computational method is now available that combines (i) potential functions that model a protein sequence’s compatibility with a structure, and (ii) fast optimization tools that can search the huge number of sequence possibilities (Dahiyat, 1999). This method was used, for example, to predict seven stabilizing point mutations, combination of which enhanced the unfolding temperature of the β1 domain of streptococcal protein G from 81°C to >100°C (Malakauskas and Mayo, 1998).

Recently, we presented a new semi-rational ‘consensus’ approach for increasing the thermostability of proteins (Lehmann *et al.*, 2000). The approach is based on the comparison of amino acid sequences of homologous proteins and the subsequent calculation of a consensus amino acid sequence using one of the available standard programs. Applying this approach to the family of fungal phytases, which at that time consisted of sequences from 13 different ascomycetes (Table I), resulted in a consensus phytase that exhibited a 15–22°C increase in its unfolding temperature compared to each of its parent phytases. The catalytic properties of the novel consensus phytase-1 resembled most those of *Emericella nidulans* and *Aspergillus fumigatus* phytase. A possible explanation—based on statistical thermodynamics—as to why more frequently occurring amino acids at a given position of an amino acid sequence alignment have a larger stabilizing effect than less frequently occurring amino acids, has been provided by Steipe *et al.* (Steipe *et al.*, 1994). This approach of using sequence comparisons to predict *individual* thermostabilizing ‘consensus’ amino acid substitutions, and the subsequent combination of experimentally proven thermostabilizing single mutations in a multiple mutant have been employed successfully for improving the thermostability of immunoglobulin domains (Ohage *et al.*, 1997, 1999; Ohage and Steipe, 1999; Wirtz and Steipe, 1999), an SH3 domain of a tyrosine kinase (Maxwell and Davidson, 1998), the tumor suppressor p53 DNA binding domain (Nikolova *et al.*, 1998), GroEL minichaperones (Wang *et al.*, 1999, 2000), as well as a WW domain (Jiang *et al.*, 2001).

Rather than to evaluate the theoretical basis of the consensus concept for thermostability engineering of proteins, the experiments outlined below were designed (i) to test empirically whether, through refinements of the method, still larger increases in thermostability than in our previous study (Lehmann *et al.*, 2000) are possible, and (ii) to evaluate the

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Table I. Source organisms of the genes, databank accession numbers and vote weights used for the calculation of the respective consensus phytases

Phytase sequence from	Databank accession No.	Vote weights			
		Consensus phytase-1	Conbasidio phytase	Consensus phytase-10	Consensus phytase-11
A.terreus 9A-1	pir:CAA02943	0.5	–	0.5	0.5
A.terreus cbs116.46	tre_fun:o00100	0.5	–	0.5	0.5
A.niger ssp. *awamori*	tre_fun:o00085	0.33	–	0.5	0.33
A.niger T213	–	0.33	–	–	0.33
A.niger NRRL3135	sw:P34752	0.33	–	0.5	0.33
A.fumigatus ATCC 13073	tre_fun:o00092	0.2	–	0.2	0.2
A.fumigatus ATCC 32722	–	0.2	–	0.2	0.2
A.fumigatus ATCC 58128	–	0.2	–	0.2	0.2
A.fumigatus ATCC 26906	–	0.2	–	0.2	0.2
A.fumigatus ATCC 32239	–	0.2	–	0.2	0.2
E.nidulans	sw:o00093	1.0	–	1.0	1.0
T.thermophilus	tre_fun:o00096	1.0	–	1.0	1.0
M.thermophila	tre_fun:o00107	1.0	–	1.0	1.0
T.lanuginosus	geneseqp:W27384	–	–	1.0	1.0
P.lycii	geneseqp:W62858	–	1.0	–	0.2
cf. Ceriporia PhyA1	geneseqp:W62859	–	0.5	–	0.2
cf. Ceriporia PhyA2	geneseqp:W62860	–	0.5	–	0.2
A.pediades	geneseqp:W62857	–	1.0	–	0.2
T.pubescens	geneseqp:W62861	–	1.0	–	0.2
Conbasidio phytase	–	–	–	1.0	–

The vote weights of the first 13 sequences were selected in order to give each *species* rather than each sequence the same weight in the calculation. Because the basidiomycete phytases form a distinct subgroup among the microbial phytases and are quite distantly related to the ascomycete phytases that were already used successfully in the calculation of consensus phytase-1, the impact of the basidiomycete phytase sequences on the calculation of the improved consensus phytase sequence(s) described here was limited by assigning a total vote weight of 1 to them.

contribution to the protein's thermostability of 'ambiguous' consensus residues, i.e. of residues for which calculation of the consensus amino acid was sensitive to the selection and weighting of the sequences used in the alignment. With these aims in mind, we added more phytase sequences to the original alignment, calculated three more consensus phytase sequences, and investigated the thermostability effects of almost every suggested amino acid replacement as single mutations in consensus phytase-1. Combination of the most favorable mutations resulted in a further 12.4°C increase in unfolding temperature over the original consensus phytase-1.

Materials and methods

Materials

Phytic acid (dodecasodium salt) was purchased from Sigma (St Louis, MO) and *p*-nitrophenyl phosphate from Merck (Darmstadt, Germany). Unless specified otherwise, *A.fumigatus* and consensus phytases were purified from the supernatants of transformed *Hansenula polymorpha* or *Saccharomyces cerevisiae* strains. Despite extensive investigations (Wyss *et al.*, 1999a,b; and unpublished data), there is no indication that the temperature optima, T_ms and specific activities of the phytases under investigation are affected by the host strain used for phytase production.

Design of the consensus phytases

The amino acid sequence alignments used for the design of the different consensus phytases (Table I) were calculated with the program PILEUP from the GCG Sequence Analysis Software Package, Release 9.0 (Devereux *et al.*, 1984) by using standard parameters (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor. The consensus sequences were calculated with the program PRETTY which is also part of the GCG package. Plurality was set at 2.0, threshold at 3, and the scoring matrix used was prettypep.cmp. The origin and the vote weight of the individual phytase amino acid sequences used in the respective calculations are shown in Table I. The impact of very similar amino acid sequences that originate from different strains of one fungal species was restricted to 1 by assigning appropriate vote weights to them. The design of the consensus sequence was restricted to the mature protein. For secretion of the protein, the first 26 amino acid residues of *Aspergillus terreus* cbs116.46 phytase were used as signal peptide and fused to the N-termini of consensus phytases-1 and -10.

Gene construction

Calculation of the DNA sequences, construction of the synthetic genes, cloning of the consensus phytase genes into the expression vector pFP, transformation of the methylotrophic yeast *H.polymorpha*, and purification of the consensus phytases expressed in *H.polymorpha* were performed as described previously (Lehmann *et al.*, 2000).

For expression in *S.cerevisiae*, the consensus phytase genes were ligated into the *Eco*RI site of the plasmid RO11, which contains a shortened version of the constitutive GAPFL (glyceraldehyde-3-phosphate dehydrogenase) promoter and the terminator of the *pho5* gene from *S.cerevisiae*, as described by Janes *et al.* (Janes *et al.*, 1990). The correct orientation of the genes was checked by PCR. Transformation of *S.cerevisiae* strains (e.g. INVSc1; Invitrogen, Carlsbad, CA) was done according to Hinnen *et al.* (Hinnen *et al.*, 1978). Single colonies harboring the phytase gene under the control of the GAPFL promoter were picked and cultivated in 5 ml of dextrose medium lacking uracil [SD-(–ura); Sherman *et al.*, 1986] at 30°C under vigorous shaking (250 r.p.m. LAB-Shaker, Kühner AG, Basel, Switzerland) for 1 day. The pre-culture was then added to 500 ml of YPD medium (Sherman *et al.*, 1986) and grown under the same conditions. After 4 days of

incubation, the culture broth was centrifuged (8000 *g*, 15 min, 5°C) to remove the cells, and the supernatant was concentrated by way of Ultrafiltration in Amicon 8400 cells (PM30 membranes; Grace AG, Wallisellen, Switzerland) and Ultrafree-15 centrifugal filter devices (Biomax-30K; Millipore, Bedford, MA). The concentrate (10 ml) was desalted on a 40 ml Sephadex G-25 Superfine column (Amersham Pharmacia Biotech, Freiburg, Germany), with 10 mM sodium acetate, pH 5.0, serving as elution buffer. The desalted sample was brought to 2 M $(NH_4)_2SO_4$ and directly loaded onto a 1 ml butyl-Sepharose 4 Fast-Flow hydrophobic interaction chromatography column (Amersham Pharmacia Biotech) which was eluted with a linear gradient from 2 to 0 M $(NH_4)_2SO_4$ in 10 mM sodium acetate, pH 5.0. Phytase was eluted in the flow-through, concentrated and loaded on a 120 ml Sephacryl S-300 gel permeation chromatography column (Amersham Pharmacia Biotech). The consensus phytases thus purified eluted as homogeneous symmetrical peaks and were shown by SDS–PAGE to be ~95% pure.

Site-directed mutagenesis

Mutations were introduced using the Quick Exchange™ Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) with appropriate primers. Plasmids harboring the desired mutation(s) were identified by DNA sequence analysis.

Activity measurements

Standard phytase activity assays were performed as described previously (Wyss *et al.*, 1999b) with 5 mM phytic acid as substrate at pH 5.0 and 37°C and by measuring the release of inorganic phosphate. One unit of activity is defined as the amount of enzyme that releases 1 µmol of phosphate per minute. pH–activity profiles were recorded by measuring phytase activity at 37°C in appropriate buffer solutions at pH values between 2.5 and 9.0 (Wyss *et al.*, 1999b). Substrate specificity was addressed by measuring the specific activities of a given phytase with a range of phosphate compounds. For that purpose, phytic acid in the standard phytase assay was replaced with 5 mM concentrations of the phosphate compounds listed in the legend to Figure 3. For determination of the temperature optima, enzyme and substrate solution (100 µl each) were pre-incubated separately for 5 min at a series of temperatures between 37 and 85°C and then mixed. After 15 min of incubation, the reaction was stopped and the amount of liberated inorganic phosphate determined according to the standard activity assay.

Differential scanning calorimetry (DSC)

For determination of the unfolding temperature (T_m), the proteins purified from *H.polymorpha* culture supernatants were extensively dialyzed against 10 mM sodium acetate, pH 5.0, and concentrated to 50–60 mg/ml. DSC experiments were performed on a Mettler DSC 821e heat flux calorimeter equipped with the STARe control and evaluation software (Version 4.00). Tightly sealed 40 µl aluminum crucibles were used for the protein samples, while the reference cell was empty. The instrument was calibrated using indium as standard. The sample was cooled down from room temperature to 5°C, the starting temperature of the experiment. After equilibration for 5 min at this temperature, a constant heating rate of 10°C/min was applied up to 90–95°C.

Other methods

Protein concentrations were determined using the enzyme extinction coefficients at 280 nm calculated according to Pace *et al.* (Pace *et al.*, 1995). One absorption unit (1 OD) at 280 nm corresponds to 1.10 mg/ml of consensus phytase-1 and 1.04 mg/ml of consensus phytase-10.

Results

Calculation of consensus phytases-10 and -11

Previously, we calculated a consensus amino acid sequence from 13 homologous fungal phytases from three *Aspergillus niger* isolates, two isolates of *A.terreus*, five isolates of *A.fumigatus*, and one isolate each of the ascomycete fungi *E.nidulans*, *Talaromyces thermophilus*, and *Myceliophthora thermophila* (Table I). A synthetic gene was constructed and recombinantly expressed. Surprisingly, consensus phytase-1 was 15–26°C more thermostable than all parent phytases used in its design (Lehmann *et al.*, 2000). In the present study, the consensus approach was refined by adding more fungal phytase sequences to the alignment.

In the case of consensus phytase-10, two more sequences were added: (i) the phytase sequence from the ascomycete *Thermomyces lanuginosus* (Berka *et al.*, 1998); (ii) the conbasidio phytase sequence, which itself is the consensus sequence of five phytase sequences from the basidiomycete fungi *Peniophora lycii*, *Trametes pubescens*, *Agrocybe pediades*, and *cf. Ceriporia* (Lassen *et al.*, 2001). The subsequent calculation of the new consensus phytase-10* sequence by PRETTY left 21 positions undefined (shown in small letters in Figure 1). Nine of these positions (46, 78, 265, 277, 297, 356, 366, 393, 423) were filled by the respective amino acid obtained from a calculation in which the conbasidio phytase sequence was omitted and the threshold was lowered from 3 to 2. At positions 18, 85, 130, 163, 166, 167 and 248, for which also in the latter calculation no consensus amino acid was defined, the corresponding amino acid residue of consensus phytase-1 was chosen. For the remaining five open positions (165, 181, 197, 223, 268), an amino acid occurring at those alignment positions in one of the wild-type phytases, but not the amino acid of consensus phytase-1, was selected arbitrarily.

The final consensus phytase-10 differed in 32 amino acid residues from consensus phytase-1 (Figure 1). Eight of those differences (at positions 46, 165, 181, 197, 223, 268, 297 and 356) originated from the filling of undefined positions as described above. Eight other differences were caused by the addition of the phytase sequence of *T.lanuginosus* to the alignment (35, 111, 174, 244, 254, 373, 381, 392). At positions 71, 191, 215, 236, 260, 343, 414 and 440, the inclusion of the conbasidio phytase sequence caused the amino acid replacement. At four positions (31, 135, 228, 341), the introduction of either the *T.lanuginosus* phytase sequence or the conbasidio phytase sequence altered the outcome of the calculation to the new residue. Both phytase sequences were necessary to change the consensus residue at positions 47 and 211. The new amino acid at position 164 was caused by an altered alignment, in which the gaps occurring in the *A.fumigatus* and *E.nidulans* phytase sequences were repositioned (alignment not shown). At position 306 of consensus phytase-1, a non-consensus residue had been chosen deliberately. In consensus phytase-10, however, the actually calculated consensus residue was used.

The differences above also might have been caused by the increased threshold used for the PRETTY session. However, changing the threshold from 2 to 3 and leaving all other conditions unchanged resulted in a consensus phytase-$10^{threshold3}$ sequence that differed in eight positions (H6S,

L191T, V204A, S250-, Q269A, -297V, -366I, K421T; a dash means that no amino acid was calculated by PRETTY) from the consensus phytase-10* sequence (the actual sequence calculated by PRETTY that still contains open positions; see above); however, none of those positions matched one of the actual amino acid changes discussed above.

In the calculation of consensus phytase-11, all five basidiomycete phytase sequences rather than the conbasidio

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                      4                                                   53
Conbasidio phytase    -S-P-R-TAA QLPIP-Q-Q- -WSPYSPYFP VA-Y-A..P. .PAGCQI-QV
Consensus phytase-11  NSHSCDTVD- GYQC-PEISH LWGQYSPFFS LADESAISPD VPKGCRVTFV
Consensus phytase-1   NSHSCDTVDG GYQCFPEISH LWGQYSPYFS LEDESAISPD VPDDCRVTFV
Consensus phytase-10  NSHSCDTVDG GYQCfPEISH LWGQYSPFFS LADESAISPD VPkGCRVTFV
                                                   $    *           #^

                      54                                                 103
Conbasidio phytase    NIIQRHGARF PTSGAATRIQ AAVAKLQSA- --TDPKLDFL -N-TY-LG-D
Consensus phytase-11  QVLSRHGARY PTSSKSKKYS ALIERIQKNA T-FKGKYAFL KTYNYTLGAD
Consensus phytase-1   QVLSRHGARY PTSSKSKAYS ALIEAIQKNA TAFKGKYAFL KTYNYTLGAD
Consensus phytase-10  QVLSRHGARY PTSSKSKKYS ALIeAIQKNA TaFKGKYAFL KTYNYTLGAD
                                        %

                      104                                                152
Conbasidio phytase    DLVPFGA-QS SQAGQEAFTR YS-LVS-DNL PFVRASGSDR VVDSATNWTA
Consensus phytase-11  DLTPFGENQM VNSGIKFYRR YKAL.ARNIV PFVRASGSDR VIASAEKFIE
Consensus phytase-1   DLTPFGENQM VNSGIKFYRR YKAL.ARKIV PFIRASGSDR VIASAEKFIE
Consensus phytase-10  DLTPFGEQQM VNSGIKFYRR YKAL.ARkIV PFVRASGSDR VIASAEKFIE
                             *                           $

                      153                                                202
Conbasidio phytase    GFA-A..... ..S-NT--P- L-VILSE-G. .NDTLDDNMC P-AGDS...D
Consensus phytase-11  GFQSAKLADP -A--HQASPV INVIIPEGSG YNNTLDHGLC TAFEDSTLGD
Consensus phytase-1   GFQSAKLADP GSQPHQASPV IDVIIPEGSG YNNTLDHGTC TAFEDSELGD
Consensus phytase-10  GFQSAKLADP gAnphQASPV INVIIPEGaG YNNTLDHGLC TAFEeSELGD
                                  1#         *      #          %      #

                      203                                                252
Conbasidio phytase    PQ-N-WLAVF APPITARLNA AAPGANLTD- DA-NL--LCP FETVS-E-..
Consensus phytase-11  DAEANFTAVF APPIRARLEA -LPGVNLTDE DVVNLMDMCP FDTVARTSDA
Consensus phytase-1   DVEANFTALF APAIRARLEA DLPGVTLTDE DVVYLMDMCP FETVARTSDA
Consensus phytase-10  DVEANFTAVF APPIRARLEA hLPGVNLTDE DVVNLMDMCP FDTVArTSDA
                              ^    %        #    $        %        *

                      253                                                302
Conbasidio phytase    ..-S-FCDLF -PEEF-AF-Y -GDLDKFYGT GYGQPLGPVQ GVGYINELLA
Consensus phytase-11  TQLSPFCDLF TADEW-QYDY LQSL-KYYGY GAGNPLGPAQ GVGF-NELIA
Consensus phytase-1   TELSPFCALF THDEWRQYDY LQSLGKYYGY GAGNPLGPAQ GVGFANELIA
Consensus phytase-10  TQLSPFCDLF THdEWiQYDY LQSLgKYYGY GAGNPLGPAQ GVGFvNELIA
                       *     %        #                               #

                      303                                                352
Conbasidio phytase    RLT-QAVRDN TQTNRTLDSS P-TFPLNRTF YADFSHDNQM VAIFSAMGLF
Consensus phytase-11  RLTHSPVQDH TSTNHTLDSN PATFPLNATL YADFSHDNTM VSIFFALGLY
Consensus phytase-1   RLTRSPVQDH TSTNHTLDSN PATFPLNATL YADFSHDNSM ISIFFALGLY
Consensus phytase-10  RLTHSPVQDH TSTNHTLDSN PATFPLNATL YADFSHDNTM VSIFFALGLY
                         2                                     $  %

                      353                                                402
Conbasidio phytase    NQSAPLDPS- PDP.NRT..W VTSKLVPFSA RMVVERL-C- G..T--VRVL
Consensus phytase-11  NGTKPLSTTS VESI-ETDGY AASWTVPFAA RAYVEMMQCE AEKEPLVRVL
Consensus phytase-1   NGTAPLSTTS VESIEETDGY SASWTVPFGA RAYVEMMQCQ AEKEPLVRVL
Consensus phytase-10  NGTkPLSTTS VESiEETDGY AASWTVPFAA RAYVEMMQCE aEKEPLVRVL
                         #                  *       *           *

                      403                                        444
Conbasidio phytase    VNDAVQPLEF CGGD-DG-CT LDAFVESQ-Y AREDGQGDFE KCFAT
Consensus phytase-11  VNDRVVPLHG CGVDKLGRCK LDDFVEGLSF ARSG..GNWA ECFA.
Consensus phytase-1   VNDRVVPLHG CAVDKLGRCK RDDFVEGLSF ARSG..GNWA ECFA.
Consensus phytase-10  VNDRVVPLHG CGVDKLGRCK rDDFVEGLSF ARSG..GNWE ECFA.
                                  %                             %
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phytase sequence were included in the alignment, however with a vote weight of 0.2 each to limit the impact of this sequence group in total to a vote weight of 1. Consensus phytase-11 contains 19 differences in comparison to consensus phytase-10*. Most of the changes affect positions for which no amino acid was defined in the calculation of either consensus phytase-11 (G13-, E367-) or consensus phytase-10* (-46K, -77E, -130N, -167H, -181S, -197D, -248R, -265D, -356K, -366I, -393A, -423L). At five positions, the procedure resulted in an amino acid replacement (Q111N, E199T, V204A, H264A, E440A).

While no thermostability data are available for consensus phytase-11 which only is a theoretical construct, consensus phytase-10 displayed a 7.4 and 9.0°C increase in unfolding temperature and temperature optimum, respectively, in comparison to consensus phytase-1 (Figure 2 and Table II).

Contribution of individual residues to consensus phytase thermostability as tested by site-directed mutagenesis

The impact on thermostability of most of the 38 residues that differed between consensus phytase-1 on one hand and consensus phytases-10 and/or -11 on the other hand was tested by site-directed mutagenesis in consensus phytase-1 (Table III). In addition, a number of substitutions were made that were based on a comparison with the basidiomycete phytases (B in Table III). Some of the introduced amino acid residues were non-consensus residues, but were present in at least one of the parent phytases (* in Table III). Changes in thermostability were measured by recording temperature–activity profiles with the supernatants of the respective *S.cerevisiae* transformants.

Stabilizing, neutral and destabilizing mutations were found, but none of them affected phytase thermostability by >3°C in the test system used. Among the 33 investigated amino acid substitutions to ‘improved’ consensus residues (present in consensus phytases-10 and/or -11), 11 were stabilizing, eight were neutral, and 14 were destabilizing, thus yielding a success rate of 33%. In sharp contrast, none of the 10 mutations to non-consensus residues (B and * in Table III) was stabilizing, while four were neutral and six were destabilizing. Most of the stabilizing mutations (eight out of 11) are located on the surface of the protein; four are part of an α-helix, but none was found inside a β-sheet. Among the destabilizing mutations, six are buried, and 14 are mainly located on the surface (Table III). Out of the 10 amino acid substitutions affecting buried residues, three were stabilizing (30% success rate). On the other hand, eight of the 15 amino acid replacements affecting surface residues proved to be stabilizing (53% success rate), suggesting that surface residues are more promising targets for thermostability engineering than buried residues. These results and the estimation that the success rate of random mutagenesis is on the order of 1% (Christians *et al.*, 1999) confirm the power of the consensus concept to predict thermostabilizing mutations. Furthermore, our data strengthen the observation already made by others (van den Burg *et al.*, 1994; Giver *et al.*, 1998; Hoseki *et al.*, 1999; Zhao and Arnold, 1999; Miyazaki *et al.*, 2000) that a majority of identified stabilizing mutations in wild-type proteins affect surface residues.

Even though only 28 of the 32 amino acid differences between consensus phytase-1 and consensus phytase-10 were tested individually by site-directed mutagenesis, the fact that consensus phytase-10 is 7.4°C more thermostable than consensus phytase-1 somewhat contrasts with the finding of an equal number (i.e. 10 each) of stabilizing and destabilizing amino acid substitutions. This may indicate that the absolute effects of the stabilizing amino acid substitutions are larger than those of the destabilizing ones, or that at least some

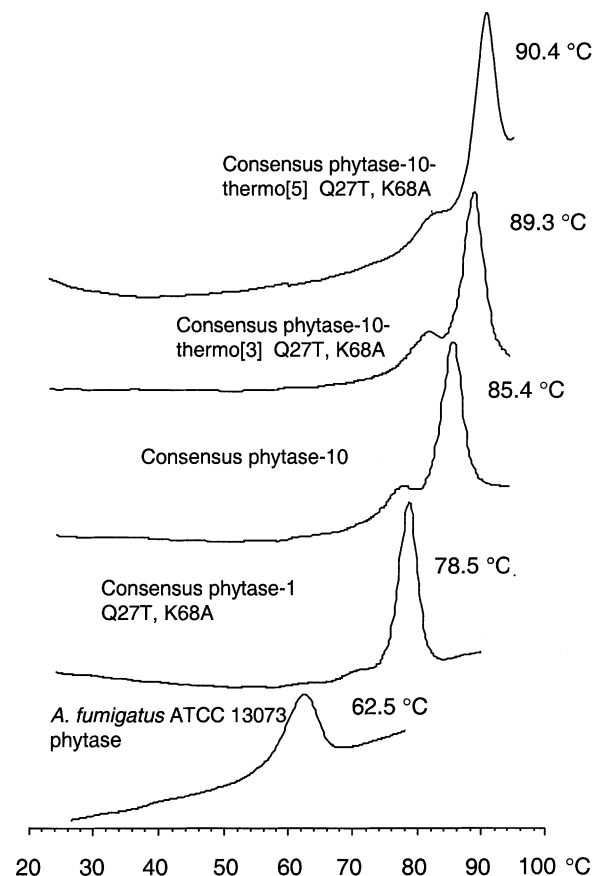

Fig. 2. DSC scans of consensus phytase-10-thermo[5] Q27T, K68A, consensus phytase-10-thermo[3] Q27T, K68A, consensus phytase-10, consensus phytase-1 Q27T, K68A and *A.fumigatus* ATCC 13073 phytase. For determination of the unfolding temperature, the proteins purified from *H.polymorpha* culture supernatants were extensively dialyzed against 10 mM sodium acetate, pH 5.0, and concentrated to 50–60 mg/ml. DSC was performed as described in Materials and methods. A constant heating rate of 10°C/min was applied up to 90–95°C.

Fig. 1. Amino acid sequence alignment of consensus phytase-1, consensus phytase-10, consensus phytase-11, and conbasidio phytase starting with residue 4. Dashes in consensus phytase-11 and conbasidio phytase represent positions for which no amino acid was determined by the procedure outlined in Materials and methods. In the case of consensus phytases-1 and -10, such open positions were filled arbitrarily; the respective amino acids are printed in lower case in the consensus phytase-10 sequence. A period stands for a gap in the sequence. The following symbols indicate the reasons for the amino acid exchanges in consensus phytase-10 relative to consensus phytase-1: #, open position that was filled arbitrarily with a residue differing from the one occurring at the respective position of consensus phytase-1; *, alteration relative to consensus phytase-1 due to inclusion of *T.lanuginosus* phytase in the alignment; %, alteration relative to consensus phytase-1 due to inclusion of the basidiomycete phytases in the alignment; $, alteration relative to consensus phytase-1 due to (*) *or* (%); ∧, alteration relative to consensus phytase-1 due to (*) *and* (%); ‘1’, alteration relative to consensus phytase-1 due to an optimized alignment; ‘2’, at position 306, residue R chosen arbitrarily in consensus phytase-1 was replaced with the consensus residue H in consensus phytase-10.

Table II. Temperature optima and unfolding temperatures (T_m) of consensus phytases

Phytase variant	Temperature optimum (°C)	Unfolding temperature T_m (°C)
Wild-type ascomycete phytases	45–55[a]	55–63[a]
T.lanuginosus phytase	65[b]	69[b]
Wild-type basidiomycete phytases	40–60[c]	48–60[c]
Consensus phytase-1	71[a]	78.0[a]
Consensus phytase-1 Q27T	n.d.	78.9
Consensus phytase-1-thermo[8] Q27T	78	84.7
Consensus phytase-1-thermo[8] Q27T, K68A	n.d.	85.7
Consensus phytase-1-thermo[11] Q27T	n.d.	88.5
Consensus phytase-1-thermo[11] Q27T, K68A	n.d.	88.0
Consensus phytase-10	80	85.4
Consensus phytase-10-thermo[3] Q27T	82	88.6
Consensus phytase-10-thermo[3] Q27T, K68A	82	89.3
Consensus phytase-10-thermo[5] Q27T, K68A	n.d.	90.4
A.fumigatus ATCC 13073 phytase Q27T	55[a]	62.5[a]
A.fumigatus 13073 phytase^{thermo}	60[a]	67.0[a]
A.fumigatus 13073 phytase^{hythermo}	63	n.d.
A.fumigatus 13073 phytase^{hythermo} K68A	63	n.d.

The temperature optima and unfolding temperatures were determined as described in Materials and methods. n.d., not determined.
[a]Data from Lehmann *et al.* (Lehmann *et al.*, 2000).
[b]Data from Berka *et al.* (Berka *et al.*, 1998).
[c]Data from Lassen *et al.* (Lassen *et al.*, 2001); note that the data from Lassen *et al.* were determined under conditions yielding 6–7°C lower T_m values than in the experiments presented here (data not shown).

Table III. Effect of single amino acid exchanges on the thermostability of consensus phytase-1

Stabilizing			Neutral		Destabilizing		
E35A[8,11] (10, 11, B)	S, C	+	D46A (B)	S, C	Y31F (10,11)	B, C	–
D46K[11] (10, 11)	S, C	+	D47G (10, 11, B)	S, C	V50I (B)	pB, β	–
D174N[8,11] (10, 11)	S, C	+	N111Q (10)	S, α	A71K (10, 11)	S, C	–
T191L[11] (10, 11)	S, C	+ +	G163H*	S, C	A78R (11)	pB, α	–
E199T[11] (11)	S, C	+ +	S164A (10, 11)	S, C	K130N (11)	S, C	–
E244D[8,11] (10, 11)	pB, α	+	T191V*	S, C	I135V (10, 11, B)	B, β	– –
R268I[8,11] (10)	S, α	+	T228N (10, 11, B)	S, C	G180A*	S, C	– –
R306H[8,11] (10, 11)	S, α	+	Y236N (10, 11, B)	pB, α	G182S*	S, C	–
S341T[8,11] (10, 11)	B, α	+ +	A260D (10, 11, B)	S, C	A194V*	S, C	–
A356K[8,11] (10, 11)	S, C	+	A297V (10)	B, α	V204A (11)	S, α	– –
G381A[8,11] (10, 11)	B, C	+ +	K422T (B)	S, β	L211V (10, 11, B)	S, C	–
			A440E (10, B)	S, C	A215P (10, 11, B)	S, C	– –
					E254Q (10, 11)	S, C	–
					H264A (11)	S, α	–
					Q269A (B)	S, α	–
					I343V (10, 11, B)	B, C	–
					S373A (10, 11)	B, C	– –
					Q392E (10, 11)	S, C	–
					A414G (10, 11, B)	S, C	– –
					E428R*	S, C	– –

+ and –, changes in thermostability up to 1°C; + + and – –, changes in thermostability between 1 and 3°C; 10, 11, B, amino acid exchanges derived from consensus phytase-10 (10), consensus phytase-11 (11) or from conbasidio phytase (B); *, ambiguous residues that were changed arbitrarily to amino acids occurring at this position in at least one of the other fungal phytases; [8], amino acid exchanges that were combined in consensus phytase-1-thermo[8]; [11], amino acid exchanges that were combined in consensus phytase-1-thermo[11]. Furthermore, the location of the residue (S, surface; pB, partially buried; B, buried) and the secondary structure element the residue is part of (C, coil; α, α-helix; β, β-sheet) are indicated.

consensus amino acids provided synergistic rather than additive thermostabilization effects.

It might be objected that the higher thermostability of consensus phytase-10 as compared to consensus phytase-1 may be due to inclusion of a slightly more stable phytase (from *T.lanuginosus*) in the alignment rather than to the alignment of *more* amino acid sequences which should make determination of the consensus amino acids more reliable. However, out of the 10 amino acid substitutions depending on *T.lanuginosus* phytase, only three were stabilizing (30% success rate), while two were neutral and five were destabilizing. Therefore, the *T.lanuginosus* phytase sequence is not likely to skew the analysis.

Additive effect of stabilizing mutations in consensus phytase-1

Eight (E35A, D174N, E244D, R268I, R306H, S341T, A356K, G381A) and 11 (E35A, D46K, D174N, T191L, E199T, E244D, R268I, R306H, S341T, A356K, G381A) of the stabilizing mutations listed in Table III were jointly introduced into consensus phytase-1, yielding variants termed consensus phytase-1-thermo[8] and consensus phytase-1-thermo[11], respectively. In addition, in some constructs, the Q27T and

K68A mutations were introduced, which have virtually no or only a minor effect on the thermostability of the protein, but increase the specific activity and broaden the pH optimum (data not shown). The 6.7 and 10.5°C increases in T_m for consensus phytase-1-thermo[8] Q27T and -thermo[11] Q27T relative to consensus phytase-1 (Table II) demonstrate that the thermostabilizing effects of the individual mutations are additive.

Thermostabilization of consensus phytase-10 through back-mutation of destabilizing residues

Not all amino acid exchanges introduced into consensus phytase-10 relative to consensus phytase-1 had a stabilizing effect as evidenced by site-directed mutagenesis in consensus phytase-1 (Table III). Therefore, the most destabilizing of these residues were reverted to the original (consensus phytase-1) residues. In consensus phytase-10-thermo[3], residues K71, V135 and A373 were replaced by A, I and S, respectively. These alterations yielded a 3.6°C increase in T_m, from 85.4 to 89.0°C (Figure 2 and Table II). Reversion of another destabilizing residue (G414A) and introduction of the stabilizing mutation E199T (suggested by consensus phytase-11) led to consensus phytase-10-thermo[5] which displayed a further increased T_m of 90.4°C (Figure 2 and Table II). A215P had a destabilizing effect in consensus phytase-1. However, the reverse mutation in consensus phytase-10 did not provide increased thermostability (data not shown). Again, the Q27T and K68A mutations positively affected the catalytic properties of the enzyme (higher specific activity between pH 2.5 and 7) while having, if at all, only small effects on the thermostability of the protein (data not shown).

Thermostabilizing effects of a subset of the consensus residues when introduced into A.fumigatus *phytase*

To evaluate the stabilizing effect of a subset of clear consensus residues in a different background, six positions in the phytase amino acid sequence alignment were selected that are dominated each by a clear consensus residue, but at which *A.fumigatus* phytase contains a rare non-consensus residue. Simultaneous replacement of these six non-consensus residues with the respective consensus residues (F28Y, V73I, F87Y, A220L, S242P, N271D) in the *A.fumigatus* ATCC 13073 phytase Q27T variant yielded *A.fumigatus* phytase 13073^{thermo} (Lehmann *et al.*, 2000). The latter displayed a 5°C increase in temperature optimum and a 4.5°C increase in T_m relative to *A.fumigatus* ATCC 13073 wild-type phytase (Table II). Introduction of four more amino acid exchanges (E35A, R306H, S341T, G381A) that were shown to be stabilizing as single mutations in consensus phytase-1 (Table III) yielded *A.fumigatus* phytase 13073^{hythermo}. This mutant also contained the S126N mutation which previously was shown to reduce protease susceptibility of *A.fumigatus* phytase (Wyss *et al.*, 1999a). Both *A. fumigatus* phytase 13073^{hythermo} and its K68A variant displayed a further 3°C increase in temperature optimum as compared to *A.fumigatus* phytase 13073^{thermo} (Table II).

Catalytic properties of consensus phytases

The catalytic properties of consensus phytase-1 resemble those of *E.nidulans* and *A.fumigatus* phytase (Lehmann *et al.*, 2000). Mutations Q27T/Q27L and K68A, which were previously shown to have pronounced effects on the catalytic properties of *A.fumigatus* phytase (Tomschy *et al.*, 2000, 2002), also increased the specific activity and broadened as well as shifted the pH optimum into the more acidic range in the various consensus phytases (data not shown). In addition, the Q27T and Q27T, K68A variants of consensus phytase-10-thermo[3] had an even higher specific activity over the entire pH range than the corresponding, less thermostable consensus phytase-1 variants (Figure 3). This is despite the fact that besides residue 71, none of the replaced amino acids is located in or close to the active site.

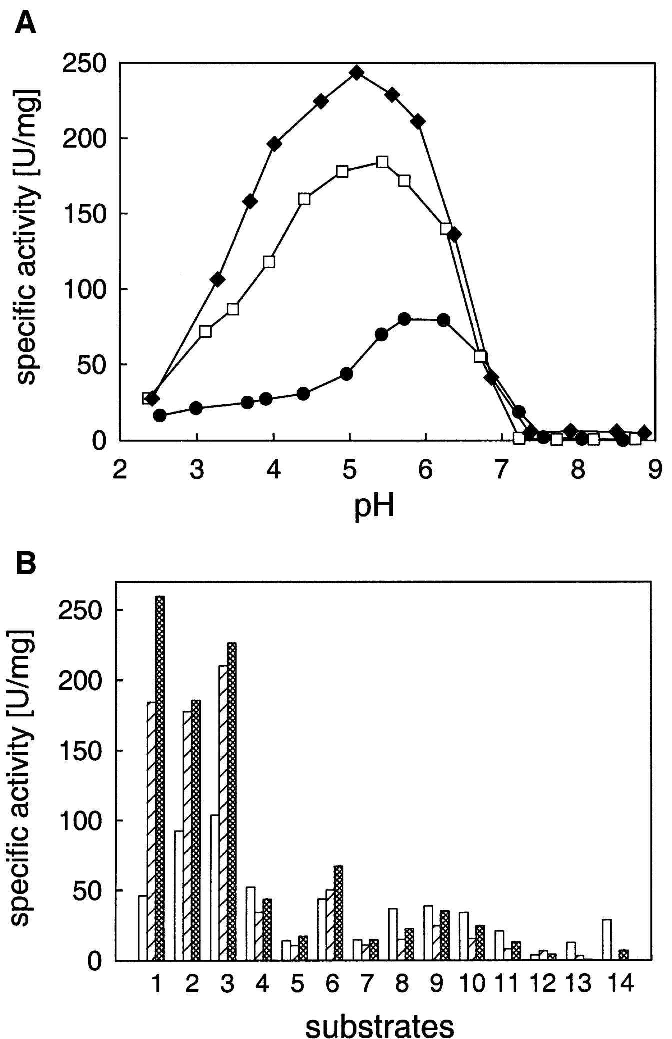

Fig. 3. Comparison of the pH–activity profiles (**A**) and of the substrate specificities (**B**) of purified consensus phytase-1 (●, open bars in B), consensus phytase-1-thermo[8] Q27T, K68A (□, hatched bars), and consensus phytase-10-thermo[3] Q27T, K68A (◆, checkered bars). pH–activity profiles were recorded by measuring phytase activity at 37°C in appropriate buffer solutions at pH values between 2.5 and 9.0. Substrate specificity was addressed by measuring the specific activities of a given phytase with a range of phosphate compounds (1, phytic acid; 2, *p*-nitrophenyl phosphate; 3, phenyl phosphate; 4, fructose-1,6-bisphosphate; 5, fructose-6-phosphate; 6, glucose-6-phosphate; 7, ribose-5-phosphate; 8, DL-glycerol-3-phosphate; 9, glycerol-2-phosphate; 10, 3-phosphoglycerate; 11, phosphoenolpyruvate; 12, AMP; 13, ADP; 14, ATP). For a comparison of the profiles with those of wild-type phytases see Wyss *et al.* (Wyss *et al.*, 1999) and Lehmann *et al.* (Lehmann *et al.*, 2000).

Discussion

Previously, we designed a novel consensus amino acid sequence, not occurring in nature, from the sequences of 13 homologous fungal phytases. Surprisingly, this consensus phytase-1 had a 15–26°C higher thermostability than its

parental phytases (Lehmann *et al.*, 2000). To further test the general validity of the consensus approach for thermostability engineering of proteins, the amino acid sequence alignment was complemented with a phytase sequence from *T.lanuginosus* and with five phytase sequences from four different basidiomycete fungi. The amended alignments were used to calculate two further consensus phytase amino acid sequences (consensus phytases-10 and -11). Consensus phytase-10 differs in 32 amino acid residues from the original consensus phytase-1 and displayed an unfolding temperature that is another 7.4°C higher than that of consensus phytase-1.

When most of the 32 differing residues were tested as single mutations in consensus phytase-1, 10 replacements had a positive effect on the protein's stability, 10 had a negative effect, and eight replacements were neutral. Four residues were not tested. All amino acid exchanges affected the thermostability by <3°C in our test system. These results indicate that the observed 7.4°C increase in unfolding temperature of consensus phytase-10 relative to consensus phytase-1 is the combined effect of multiple stabilizing amino acid replacements that are distributed over the entire protein. It is, thus, not the result of a single dominant mutation that was introduced just by chance. This interpretation in all likelihood also applies to the 15–26°C increase in unfolding temperature of consensus phytase-1 over the proteins used for its design.

We have shown—in accordance with findings from Steipe *et al.* (Steipe *et al.*, 1994)—that the consensus approach does not always select the most stabilizing amino acid for a given position of an amino acid sequence alignment. As shown by site-directed mutagenesis (Table III), 10 amino acid substitutions newly introduced in consensus phytase-10 as compared to consensus phytase-1 had a destabilizing effect relative to the original amino acid residue. Combining only the amino acid exchanges in consensus phytase-1 that showed a stabilizing effect resulted in a protein, consensus phytase-1-thermo[11] (that also harbors a substitution derived from consensus phytase-11), that had a 3°C higher unfolding temperature than consensus phytase-10. Taking this approach further, we back-mutated four of the identified destabilizing residues of consensus phytase-10 and introduced one stabilizing residue derived from consensus phytase-11 (see above). The resulting protein, consensus phytase-10-thermo[5], had an unfolding temperature of 90.4°C which is another 5°C higher than that of consensus phytase-10.

It needs to be emphasized that the amino acid replacements summarized in Table III affect positions of the alignment for which the determination of a consensus residue was ambiguous for both consensus phytase-1 and consensus phytase-10, either because of high variability or because two or more amino acids are found at those positions with more or less the same frequency. However, the fact that consensus phytase-10 is more thermostable than consensus phytase-1 demonstrates that ambiguous positions can be optimized, in terms of their contribution to phytase thermostability, by including more sequences in the alignment. Alternatively, if additional sequences are not available, site-directed mutagenesis can be used to address the impact of ambiguous consensus residues on the stability of the protein, as shown in Table III.

To address whether both clear and ambiguous consensus amino acids have a more general effect on the thermostability of fungal phytases, six clear consensus residues were introduced into *A.fumigatus* phytase, either alone or in combination with four ambiguous amino acids identified in the present study to have a stabilizing effect on the consensus phytases. The resulting variants *A.fumigatus* phytase 13073^{thermo} (Lehmann *et al.*, 2000; note that by mistake, it was not mentioned in the previous publication that *A.fumigatus* phytase 13073^{thermo} also contains the Q27T mutation) and *A.fumigatus* phytase 13073^{hythermo} (this study) displayed increases in temperature optima relative to *A.fumigatus* wild-type phytase of 5 and 8°C, respectively. Thus, typical consensus residues—and even ambiguous consensus residues identified to be stabilizing—improve the intrinsic stability not only in the consensus phytase environment, but also in *A.fumigatus* phytase and most probably in other homologous phytases. This is a further indication that the consensus approach relies on more general rules that are valid beyond the family of fungal phytases.

In some respect, thermostabilization of proteins by DNA family shuffling (Ness *et al.*, 1999) and by our consensus approach resemble each other. Both methods are able to combine amino acids, the stability contribution of which is higher than average, in a new variant that exhibits increased intrinsic stability. In the case of gene shuffling, homologous genes are digested to small pieces and newly assembled by PCR. Variants that display increased intrinsic stability are then identified by screening or by applying a selective pressure. It is interesting to note that assembly statistics are likely to bias gene shuffling experiments towards consensus sequences since in any given generation the probability of extending a fragment by a consensus residue is larger than choosing a non-consensus residue. The size of the fragments to be reassembled limits the generation of possible new combinations in gene shuffling experiments. In the case of the consensus approach, no restrictions exist *per se* for the recombination of amino acid residues. Every single residue is 'optimized' independently. Thus, the consensus approach describes a clearly defined way to determine, ideally, one final sequence for a more thermostable variant that basically consists of the most frequent amino acid at every position of a sequence alignment of homologous proteins.

The consensus approach for stabilizing proteins has the following advantages: (i) a given residue is replaced only by an amino acid that has already proven its evolutionary fitness at the corresponding position of at least one of the other homologous wild-type proteins, thereby reducing the risk of deleterious mutations; (ii) no 3D-structure is required for the method to be applicable to a given protein family; (iii) no analytical assay suitable for high-throughput screening is required.

In conclusion, through a combination of our consensus approach with limited site-directed mutagenesis work, we were able to increase the unfolding temperature of phytase from 48 to 69°C, as observed for the parental wild-type fungal phytases, to up to 90.4°C for consensus phytase-10-thermo[5]. This 21–42°C increase in intrinsic thermostability is remarkable, even when compared to some of the most impressive examples of protein stabilization reported in literature: using directed evolution, subtilisin E, *p*-nitrobenzyl esterase from *Bacillus subtilis*, and a thermostable kanamycin-resistance gene product from *Thermus thermophilus* were all stabilized by 17–20°C (Hoseki *et al.*, 1999; Spiller *et al.*, 1999; Zhao and Arnold, 1999). Using a more rational approach, van den Burg *et al.* (van den Burg *et al.*, 1998) enhanced the temperature optimum of an already thermostable bacterial protease by a further 21°C. In addition to other lines of evidence, the higher

thermostability of consensus phytase-10, calculated from an amended sequence alignment, relative to consensus phytase-1 strengthens the idea that our consensus approach is generally applicable, even and well beyond phytases.

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