THE PROTEIN SOCIETY

Refolding the unfoldable: A systematic approach for renaturation of *Bacillus circulans* xylanase

Miriam P. Kötzler,[1] Lawrence P. McIntosh,[1,2,3] and Stephen G. Withers[1,2,3]*

[1]Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1
[2]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3
[3]Michael Smith Laboratories, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Received 5 April 2017; Accepted 24 April 2017
DOI: 10.1002/pro.3181
Published online 2 May 2017 proteinscience.org

Abstract: Xylanases are important polysaccharide-cleaving catalysts for the pulp and paper, animal feeds and biofuels industries. They have also proved to be valuable model systems for understanding enzymatic catalysis, with one of the best studied being the GH11 xylanase from *Bacillus circulans* (Bcx). However, proteins from this class are very recalcitrant to refolding *in vitro*. This both limits their high level expression in heterologous hosts, and prevents experimental approaches, such as peptide ligation or chemical modifications, to probe and engineer their stability and function. To solve this problem, a systematic screening approach was employed to identify suitable buffer conditions for renaturing Bcx *in vitro*. The fractional factorial screen employed identified starting conditions for refolding, which were then refined and developed into a generic protocol for renaturing preparative amounts of active Bcx in a 50–60% yield from inclusion bodies. The method is robust and proved equally proficient at refolding circularly permuted versions that carry cysteine mutations. This general approach should be applicable to related GH11 xylanases, as well as proteins adopting a similar β-jellyroll fold, that are otherwise recalcitrant to refolding *in vitro*.

Keywords: protein refolding screen; protein expression; inclusion body; enzymology; NMR spectroscopy; carbohydrate-active enzyme; *Bacillus circulans* endo-β-1,4-xylanase

Introduction

Xylan is a major component of lignocellulosic materials and amongst the most abundant polysaccharides in nature. Technologically, lignocellulosics have long-standing importance as raw materials in the pulp and paper industry.[1] More recently, they also represent a potentially renewable and sustainable source of fuels that does not compete with food production and that could replace fossil-derived counterparts. However, due to the structural complexity of

Abbreviations: Bcx, *Bacillus circulans* endo-β-1,4-xylanase; CAZy, carbohydrate active enzyme database; cp, circular permutant; DDM, *N*-dodecyl β-maltoside; FNIII, fibronectin type III module; GuHCl, guanidinium hydrochloride; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum coherence; NDSB, non-detergent sulfobetaine; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PEG, polyethylene glycol; TRIS, tris(hydroxymethyl)aminomethane.

Additional Supporting Information may be found in the online version of this article.

Statement of Impact: In this manuscript, we present a systematic approach for discovering and optimizing a protocol for the efficient *in vitro* refolding of an aggregation-prone protein. The method was applied to a bacteria xylanase that is used extensively in biotechnology and as a model system for understanding and engineering enzymatic catalysis.

Grant sponsor: The Natural Sciences and Engineering Research Council of Canada (to L.P.M. and S.G.W.), The Deutsche Forschungsgemeinschaft (to M.P.K.), The Canada Foundation for Innovation and the British Columbia Knowledge Development Fund.

*Correspondence to: Stephen G. Withers, Department of Chemistry, 2036 Main Mall, University of British Columbia, Vancouver, BC, Canada V6T 1Z1. E-mail: withers@chem.ubc.ca

lignocellulose, controlled degradation and depolymerization without excessive use of harsh chemicals is challenging. In the effort to meet this challenge, enzymes that hydrolyze xylan (xylanases) have been employed for decades in pulp processing, and improved strategies for enzymatic processing of lignocellulose remain of high interest for biofuel production.

The best characterized of the highly specific xylanases belong to CAZy family GH11 (Carbohydrate Active Enzymes database, http://www.cazy.org/). These enzymes are important components of xylanase mixtures currently employed in the pulp and food industry (for reviews see refs. [2–4]). The low molecular weight (~20 kDa) and high thermal and pH stability of the GH11 xylanases make them particularly interesting candidates for further optimization for use in industrial processes. In addition, the GH11 *Bacillus circulans* β-1,4-endoxylanase, Bcx, has proved to be an excellent model system for understanding structures and mechanisms of xylanases, as well as glycoside hydrolases in general.[5–13] The three-dimensional structures of the enzyme have been determined in complex with a variety of ligands, and numerous mutants (including circular permutants) have been made and kinetically analyzed. Detailed NMR spectroscopic studies have also been performed to provide insights into Bcx dynamics and electrostatics, with a particular emphasis on site-specific p*K*a measurements.[5–13]

Unfortunately, one limitation of this system for both fundamental enzymology studies and biotechnological applications is that an efficient refolding protocol for Bcx and other GH11 xylanases is lacking. Most likely this is a result of the highly hydrophobic nature of the GH11 sequences. Although giving rise to a very stable native β-jellyroll fold, the denatured polypeptide chains have also a notoriously strong tendency to aggregate, leading to very poor yields of correctly folded, active enzymes.[7]

Within its native context in *Bacillus circulans*, Bcx is expressed with a cleavable signal peptide that directs the protein for secretion.[5–12,14] This sequence is not needed for proper folding as Bcx lacking a secretory peptide can still be efficiently expressed in a heterologous host such as *Escherichia coli*.[14] Under mild induction conditions, the cysteine-free protein folds to its active conformation within the soluble cytosolic fraction, whereas more aggressive induction leads to the formation of inclusion bodies. Presumably this reflects the limited capacity of the bacterial proteostasis machinery, including chaperones, to prevent the aggregation of nascent protein chains and to ensure proper folding to a native structure in a cellular environment. Furthermore, even active circular permutants of Bcx can be produced using *E. coli* expression systems, demonstrating that the enzyme adopts its native fold in a manner that does not depend upon the locations of its termini.[15] Hence, a productive *in vivo* folding process must exist independently of the translation and secretion processes, implying that *in vitro* refolding of Bcx, and presumably other GH11 enzymes, to a thermodynamically favorable native state should be possible. However, *in vitro* refolding in simple solution conditions can be complicated by competing pathways that yield kinetically trapped aggregates or other misfolded species. We therefore set out to find a robust and efficient refolding protocol for Bcx. The strategy that we developed to meet this goal is general and thus should be readily adapted toward related xylanases, as well as other folding-recalcitrant proteins.

Results and Discussion

Although there are well-known guiding principles for favoring folding and minimizing aggregation of proteins *in vitro*, determining an optimal protocol for renaturation remains an empirical process. Nevertheless, insights into potential conditions for refolding of Bcx can be gleaned from a study of a proline-rich all-β-sheet fibronectin type III (FNIII) module.[16] This small protein (94 residues) adopts a very stable all-β-sheet Greek-key fold with a high contact order.[16] Similar to Bcx, FNIII is a small, globular protein that possesses a stable hydrophobic core rich in aromatic residues. FNIII refolds very rapidly (<1 s at 5°C) upon 11-fold dilution from 7 M GuHCl into a buffer containing 0.7 M GuHCl and 20 mM sodium acetate pH 5.2.[16] The denaturant may slow down the hydrophobic collapse, thereby leading to a smaller fraction of misfolded proteins. Similarly, the addition of aggregation inhibitors such as polyethylene glycol, sugars or detergents is expected to protect solvent-exposed hydrophobic patches of folding intermediates, rendering aggregation pathways less favorable.[17] This background information provides a useful starting point for discovering conditions to refold Bcx.

Fractional factorial screens reveal refolding conditions for Bcx

Fractional factorial screens are a systematic and rapid method to identify conditions for processes such as protein crystallization or refolding.[18] Rather than tediously testing numerous parameters independently, these screens involve simultaneous variation of multiple parameters in a fraction (1/2, 1/4, etc.) of the samples that would be required for a full combinatorial screen. From this, general trends favoring folding can be identified. Once coarsely defined, further optimization steps can be readily implemented.

For Bcx, a fractional factorial screen was designed to rapidly explore the effects of seven different

additives, three different detergents, and eight different pH conditions (Table SI). We modeled our screen on the general approach of Willis *et al.*,[18] which has since been implemented into several commercially available refolding kits. However, we did not use these generic screens, since they heavily focus on proteins with disulfide bonds (redox conditions) and proteins with divalent metal cations. Also, Bcx and other GH11 enzymes proved are reluctant to refolding under conditions that typically work for many other proteins.

Instead, we customized buffer additives based on the specific properties of family GH11 enzymes. We omitted reducing agents since wild-type Bcx does not contain any cysteines or disulfide bonds. Likewise, divalent cations were also omitted since none are known to bind Bcx with significant affinity. Instead we chose xylose and glycerol as additives, as well as an array of detergents and aggregation inhibitors. The former represents a partial ligand and the latter was used in the successful refolding of catalytic amounts of several Bcx mutants.[7,12] Since distinct charge states might be crucial for formation of initial contacts during folding and since Bcx has a high isoelectric point of ~9.1 yet maximum activity at pH 5.5, we tested eight different pH values ranging from 3.5 to 9.5. To stabilize the anticipated large hydrophobic patches present in the unfolded or partially folded states of the protein, three detergent conditions (0.3 mM DDM, 0.5 mM Tween-80, and 1 M NDSB-201) were also chosen.[18] Finally two concentrations each of PEG 3350 (aggregation inhibitor), sodium chloride, sucrose (osmolyte folding agent), arginine hydrochloride (aggregation inhibitor) and guanidinium chloride (GuHCl, chaotropic salt) were included in the screen.[18] These additives, detergents and buffers were combined to produce 32 fractional factorial screening solutions, listed in Table SI.

To carry out the screen, we dissolved purified native Bcx at 1 mg/mL in sodium phosphate buffer containing 6 M GuHCl at pH 6.0. Although Bcx unfolds cooperatively at a midpoint GuHCl concentration between 2 and 3 M (L.P.M., unpublished observation), the high concentration of denaturant was used with the assumption of producing a nearly monodisperse solution of fully denatured protein. Also, it may be more difficult to achieve this starting state from inclusion bodies that contain impure aggregated protein. Samples were then rapidly diluted 20-fold into each of the 32 screening solutions in a 96 multi-well plate and incubated overnight at 4°C. Note that due to the initial denaturation conditions chosen, all final samples end up containing at least 0.3 M GuHCl. The aggregation state of each sample was then evaluated by measuring its turbidity spectrophotometrically at 340 nm, 405 nm and 560 nm using a plate reader. Although common trends were seen at all wavelengths studied, the turbidity measured at 340 nm was found to be the most sensitive, and thus was used in subsequent trials. As a negative control (maximal aggregation), unfolded Bcx was diluted into wells containing buffers at all eight pH values, without any additives. As positive controls (maximal correct folding), the same amount of native enzyme was diluted into similar buffered solutions.

The 32 refolding conditions studied were found to yield the entire range of turbidities. Samples with the highest turbidity matched those of the negative controls, whereas the lowest values were on the level of that exhibited by the native Bcx solutions. The relative aggregation states of samples were calculated from these turbidity values, normalized to the lowest value of the positive controls and to the highest value of the negative controls. To validate the notion that aggregation, as measured by turbidity at 340 nm, does indeed correlate inversely with correct folding, we also determined the activities of a subset of samples. Indeed, samples with the highest turbidities had very low specific activities (<1% of the control), whereas those with low turbidities exhibited high specific activities (60–100% of the control) (Tables SI and SII). Turbidity therefore reliably reflects refolding of our Bcx samples. It is also noteworthy that turbidity measurements are simpler than alternative methods such as dynamic light scattering.

The relative aggregation levels for the sets of sample groups in each of the eight buffer (pH) conditions, along with an indication of the ranges of aggregation states, are summarized in Figure 1(A). Although acidic conditions clearly promote aggregation, the large spread of values observed reflects how some additives can reduce aggregation, even at the lower pH values. In contrast, the spread of values at pH 7.0 is narrow, reflecting generally low aggregation regardless of the additives. To assess how refolding is affected by the general presence of an additive, the means of the normalized aggregation of all samples containing that additive were plotted alongside the equivalent conditions without the additive [Fig. 1(B)]. Clearly, the presence of 0.06% w/v PEG 3350 or of 1 M GuHCl had the largest impact on lowering aggregation. An analysis of how pH affected these results revealed particularly pronounced effects at pH 5.5–7.5 (data not shown). In contrast, the presence of NaCl and xylose promoted aggregation, whereas sucrose, arginine and glycerol showed only minor effects.

Due to the 1/8 fractional design of the screen with simultaneous variation of conditions, the principal effects of one parameter can be confounded with other parameters. For example, Figure 1(C) shows the pH-dependence of refolding in the absence of any detergent and in the presence of three different detergents. The lowest means of aggregation were achieved in the presence of Tween-80 and pH

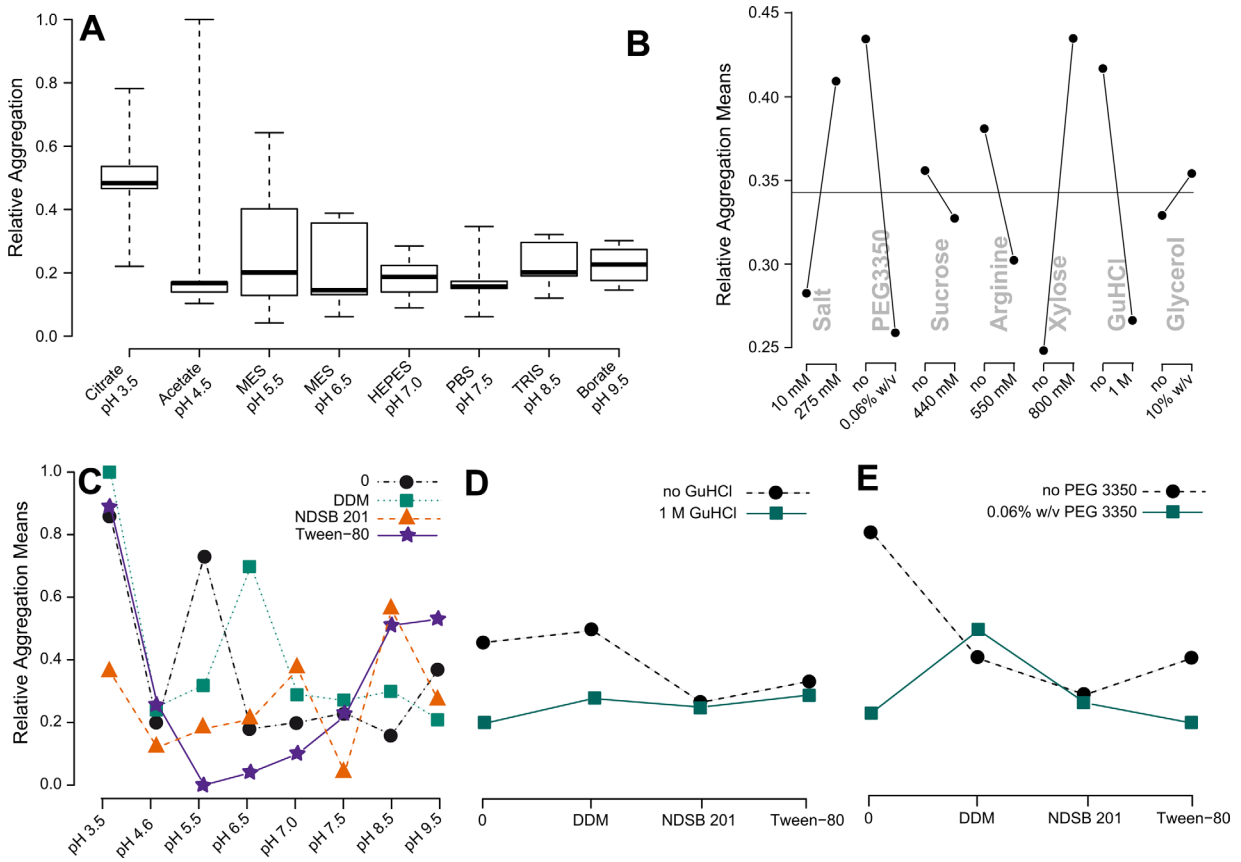

Figure 1. Results of the initial fractional factorial screening to coarsely identify refolding conditions for Bcx. The effects of pH were tested on 8 levels (8 pH values), detergent on 4 levels (3 types and no detergent) and sodium chloride, PEG 3350, sucrose, arginine, xylose, GuHCl and glycerol on 2 levels each (absence and presence). The *y*-axes indicate relative aggregation values, defined as the turbidity normalized to the highest (negative control) and the lowest (positive control) values in the screen. (A) Boxplot showing normalized aggregation for samples at different pH conditions, including samples that do not contain any additives (dark center lines are the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend to minimum and maximum values; $n = 5$ sample points). (B) Main effects for the 7 additives tested at two levels. Shown are the mean relative aggregation values for samples containing the 7 additives versus the corresponding samples lacking the additive. (C) Interaction (or co-dependence) plot showing how aggregation changes when detergent is absent (circles) or present (squares, triangles and stars) as a function of sample pH. (D) Interaction plot showing the effects of detergent on reducing aggregation in the presence and absence of GuHCl. (E) Interaction plot showing the effects of PEG 3350 on reducing aggregation as a function of detergent. Note that the lines in the interaction dot plots are a guide to the eye, connecting data points associated with one constant condition.

5.5–7.0, whereas NSDB-201 was superior outside this pH window. In contrast, the presence of DDM did not lower aggregation at most pH values. PEG 3350 and GuHCl had their strongest effects in solutions at slightly acidic pH values of 4.5–6.5 (data not shown). Figure 1(D,E) illustrates the dependence of detergent type on the two additives that exhibited the strongest principal effects, namely GuHCl and PEG 3350. The aggregation inhibitory effect of 1 M GuHCl was either not significantly enhanced or was counteracted by all of the three detergents tested. A similar trend was observed for the aggregation inhibitory effect of PEG 3350. Neither Tween-80 nor NSDB 201 further enhanced the effect, and DDM even slightly counteracted it. Likewise, PEG 3350 and GuHCl did not exhibit any co-dependence (data not shown).

In the initial screen, we identified factors that help prevent aggregation on their own (PEG 3350, GuHCl, NSDB-201 or Tween-80, pH 5.5 - 7.5). However, we were unable to determine which combination of components was optimal for establishing favorable interactions. Thus, we set up a second screen to assess the more ambiguous factors (arginine, sucrose), to refine the results (sample pH and GuHCl concentration), and to determine favorable interactions to find the optimal combination of additives. In this screen, all conditions included 0.06% w/v PEG 3350 and at least 0.5 M GuHCl because these factors were already identified as strongly beneficial. All other factors were then assessed in the background of these additives. The sample pH value was tested at four levels (MES pH 5.5, MES pH 6.5, HEPES pH 7.0 and TRIS pH 8.5) and with two

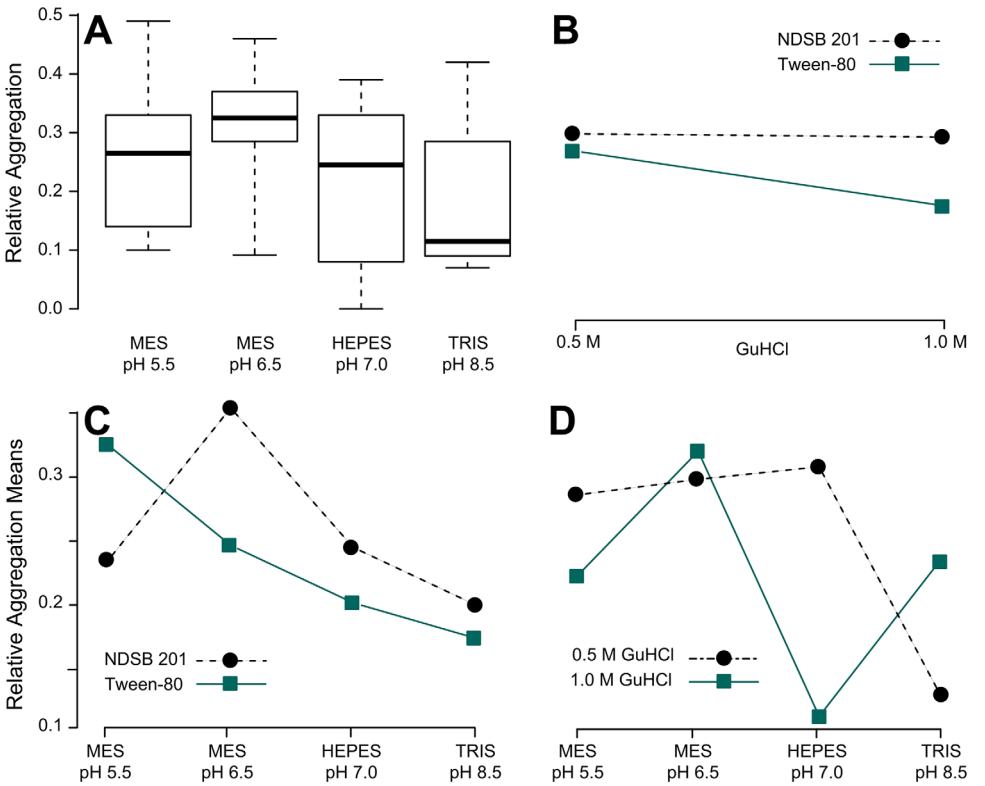

Figure 2. Results of the refinement screen for refolding of Bcx. All samples contained 0.06% w/v PEG 3350 and GuHCl at 0.5 M or higher. Sample pH was tested on 4 levels whereas detergent, GuHCl, arginine and sucrose were tested on 2 levels each. (A) Boxplot for the distribution of aggregation values at different pH values (dark center lines are the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend to minimum and maximum values; $n = 8$ sample points). (B) Interaction plot for two concentrations of GuHCl with two different detergents. (C) The pH-dependence of the detergents. (D) The pH-dependence of GuHCl on reducing aggregation. Note that the lines in the interaction dot plots are a guide to the eye, connecting data points associated with one constant condition.

different detergents (NDSB-201 and Tween-80) to determine which combination is superior. The concentration of GuHCl was tested at two different levels (0.5 and 1.0 M), whereas arginine and sucrose were tested at the same levels as in the first screen to re-evaluate their weaker effects.

The overall mean aggregation in this screen was considerably lower than in the first screen, confirming the improvement in folding conferred by the refined conditions of sample pH, and the presence of PEG 3350, GuHCl and selected detergents. The samples with minimal aggregation tended to have a pH of 8.5 or 7.0 [Fig. 2(A)]. In terms of additives, Tween-80 was slightly more effective at inhibiting aggregation than NSDB-201 in the presence of GuHCl at 1.0 M, but not 0.5 M [Fig. 2(B)]. Stated alternatively, no difference was seen in the mean effect with NSDB-201, yet samples with Tween-80 benefitted from a higher guanidine concentration. In contrast, arginine promoted aggregation under the refined conditions and sucrose had no effect (data not shown). Co-variation of sample pH and detergent revealed that the effects of Tween-80 reduced aggregation steadily from pH 5.5 to 8.5, whereas NSDB-201 acted best above or below pH 6.5 [Fig. 2(C)]. In contrast, the higher concentration of GuHCl worked best at pH 7.0, whereas lower concentrations were better matched with pH 8.5 [Fig. 2(D)]. The effect of GuHCl concentration also depended on the detergent present.

An initial coarse fractional factorial screen, followed by a more focused screen, allowed us to identify conditions that favored the folding of Bcx with minimal aggregation. Since Tween-80 is the least expensive reagent, shows improved effects in the presence of 1 M GuHCl and does not interfere with PEG 3350 [Fig. 1(E)], this detergent was chosen as a key buffer component. An optimized refolding buffer was also found to contain HEPES at pH 7.0 or Tris pH 8.5, because all chosen factors have their highest or nearly highest effect at this sample pH value. To confirm the validity of these results based on turbidity measurements, the enzymatic activities of Bcx folded under these 10 best conditions were measured (Table SI). Enzyme concentrations were determined by absorbance at 280 nm and activity was calculated relative to that of native Bcx at 20°C and pH 6.5, for which $k_{cat} = 30\ s^{-1}$. Indeed, the condition containing 0.06% w/v PEG 3350, 1.0 M GuHCl, 0.5 mM Tween-80 and 50 mM HEPES pH 7.0 yielded refolded Bcx with a relative activity of 80% of the reference.

Protein refolded under the second best condition (0.06% w/v PEG 3350, 0.5 M GuHCl, 0.5 mM Tween-80 and 50 mM TRIS pH 8.5) showed 70% activity relative to the total amount of protein, whereas that from the next best eight compositions ranged between 25% and 65% activity. These conditions contained NSDB-201 or were at a more acidic pH value. These results showed that, although an optimal refolding condition was identified, slightly different buffer conditions could also be used, if for example acidic pH was preferred because a cysteine mutant was to be refolded.

Scaled up refolding by pulsed dilution provides a 90% yield of active Bcx

Development of a scalable method for refolding requires careful consideration of the protocol for dilution of the protein from the denaturing buffer into the refolding buffer. In general, protein aggregation is strongly concentration-dependent and thus a balance between sample dilution and practical sample volume must be found. In our small scale screening assays, we use a final concentration of 50 μg/mL Bcx in 100 μL. In a previously published Bcx refolding protocol, dialysis was the chosen method for buffer exchange from the denaturing buffer to the refolding buffer.[7] However, this was probably not a good choice for two related reasons. First, large volumes of denaturing buffer were needed to maintain preparative amounts of protein at a concentration low enough to prevent aggregation. Second, although properly folded Bcx is very stable over a wide range of concentrations and solution conditions, it does have a propensity to reversibly self-associate at millimolar concentrations and elevated ionic strength buffers.[10] In contrast, when placed in non-denaturing conditions, unfolded Bcx appears to be extremely prone to aggregation, presumably due to an abundance of exposed hydrophobic residues. This requires the rapid dilution of unfolded Bcx into relatively large volumes of refolding buffer to minimize the concentrations of any fully or partially unfolded species.

Accordingly, we developed a refolding protocol involving pulsed dilution. That is, Bcx is introduced step-wise into a refolding buffer in small portions with a time period between the pulses that allows the denatured enzyme to refold at very low concentrations (<5 μg/mL).[19] The concentration of folded Bcx will increase after each pulse, but this should not impact sample aggregation. Following the same strategy as for the screen, we tested this refolding technique by adding denatured Bcx (1.0 mL of 1 mg/mL in 6 M GuHCl, 50 mM phosphate pH 6.0) dropwise to pre-cooled refolding buffer (20 mL, 4°C) with stirring to ensure rapid dilution of any unfolded protein. The sample was then incubated at 4°C overnight to precipitate any misfolded protein, allowing it to be removed by centrifugation. Absorbance at 280 nm revealed that the final protein concentration was ~50 μg/mL. The solution had a clear appearance and the ultraviolet absorption spectra did not show any turbidity due to the presence of aggregates. According to measured enzymatic activity, 90% of the total protein refolded. The higher refolding yield obtained here relative to the miniaturized screen confirms that the dilution technique is robust. The refolded Bcx was concentrated to 2.6 mg/mL and exchanged into phosphate buffer, pH 6.0. Since it still contained a significant amount of PEG 3350 and possibly Tween-80, cation exchange chromatography was used for final purification. The purified enzyme was obtained in a satisfying 86% isolated yield of refolded enzyme, relative to the <1% reported yield in previous attempts.[7]

Refolding of wild-type Bcx from inclusion bodies

We next applied our refolding protocol to protein from inclusion bodies produced by high level expression of ^{15}N-labeled Bcx in *E. coli*. Washed inclusion bodies were dissolved in denaturing buffer (7 M GuHCl, 50 mM sodium phosphate, pH 6.0) at 1 mg/mL (50 μM) and centrifuged at 30,000*g*, 4°C for 30 min. Thorough mixing and fast dissolution of the inclusion bodies was found to be crucial to obtain a solution that contains few initial protein aggregates. Indeed, at this point of our study, we discovered that even in the presence of 6 M GuHCl or 8 M urea, denatured Bcx has a tendency to form extremely stable, probably amyloid-like aggregates. This process is accelerated at acidic pH, high protein concentrations and in the presence of any remaining aggregates from undissolved inclusion bodies (data not shown). Such aggregates, which are invisible to the eye but detected by their early elution from reversed phase HPLC, reduced the refolding yield significantly in our experiments. The fresh, clarified protein solution at 1 mg/mL was therefore directly subjected to the pulsed dilution refolding protocol described above. After purification by cation exchange chromatography, 46% of the total protein subjected to refolding was recovered as soluble, active enzyme. Furthermore, the ^{15}N-HSQC spectrum of the refolded protein was essentially identical to that of a reference sample obtained from the soluble lysate of an *E. coli* expression host (Fig. 3). This confirms that the refolded protein adopts the well characterized structure of native Bcx.

Refolding of circular permutants from inclusion bodies

To demonstrate that our method is broadly applicable to other constructs of Bcx, we also refolded two of its circular permutants. These variants each have a single cysteine introduced at a solvent exposed site

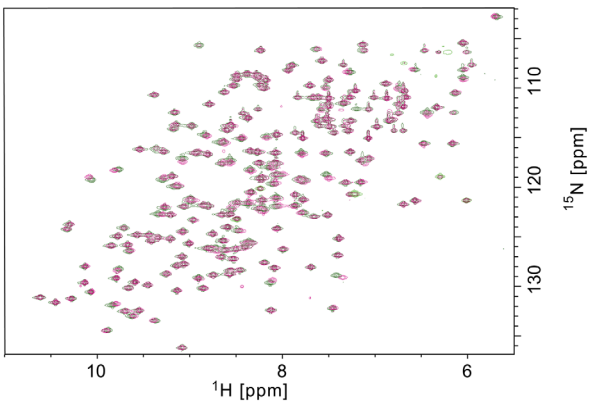

Figure 3. The ^{15}N-HSQC spectrum of Bcx refolded from inclusion bodies (pink) overlaid on the control spectrum of solubly expressed Bcx (green). The well dispersed and closely matching amide $^{1}H^{N}$–^{15}N chemical shifts confirm the correct refolding of the enzyme. Small chemical shift differences are attributed to the exact sample conditions.

that will be exploited for planned peptide ligation experiments. The enzymes adopt the same tertiary structure as wild-type Bcx,[20] but have their N-termini placed at position Tyr174 (*cp*Y174-S162C) or Pro75 (*cp*P75-T87C), respectively. Although both constructs expressed in *E. coli* at a comparable level to the wild type enzyme, the yields of soluble protein were significantly lower, especially with *cp*P75-T87C (data not shown). In the bacterial system, folding of this enzyme is likely to occur co-translationally, hence the shifted termini might impact folding efficiency. In contrast, this effect is not expected to the same extent for *in vitro* folding, since the random-coil chain should, in principle, be able to form similar initial contacts and folding intermediates for the different circular permutants. Indeed, using our optimized protocol, cpP75-T87C provided 60% and cpY174-S162C 55% isolated yield of purified, active enzyme relative to the total amount of denatured protein from inclusion bodies subjected to refolding. This stands in contrast to the negligible fraction of soluble enzyme found in the cell extract. Interestingly, yields of the three constructs (WT, cpP75-T87C and cpY174-S162C) we tested our *in vitro* refolding protocol follow the opposite trend from the yields of native enzyme *in vivo*. cpP75-T87C showed the highest yield *in vitro* (65%), yet lowest *in vivo*, whereas wild-type Bcx had the lowest *in vitro* (46%), yet highest *in vivo*.

Conclusions

In conclusion, using a fractional factorial screening approach, we developed a robust *in vitro* protocol for obtaining active refolded Bcx from an initially denatured state. The optimized protocol involves the step-wise pulsed dilution of Bcx from 1 mg/ml in 6 M GuHCl by approximately 20-fold into a refolding buffer containing 0.06% w/v PEG 3350, 1.0 M GuHCl, 0.5 mM Tween-80 and 50 mM HEPES pH 7.0 at 4°C. Our method also greatly improves the yield of Bcx variants from inclusion bodies in bacterial expression systems. Being able to refold GH11 enzymes has several experimental benefits, including the use of peptide ligation to engineer chemically modified forms of the enzyme. This work also exemplifies a general strategy for identifying conditions that favor the refolding of proteins with a high propensity for aggregation or other misfolding processes.

Materials and Methods

PCR cloning of cysteine mutants and circular permutants of Bcx

The circular permutants were generated with a Gly–Gly linker joining the native ends (Ala1 and Trp185), with cysteines at exposed sites, and with the new N-terminus introduced at the positions 75 (cpP75-T87C) or 174 (cpY174-S162C). Clones encoding these were generated from the wild type gene sequence or permuted sequences by a PCR approaches. The products were placed into a pET29b(+) vector (Novagen) using NdeI and HindIII restriction sites. The DNA and amino acid sequences of the designed circular permutants are listed in Table SIII.

Protein expression and purification

Wild-type Bcx was expressed from a pET22b plasmid (ampicillin resistance) and circular permutants from a pET29b plasmid (kanamycin resistance). Cultures of transformed *E. coli* BL21(λDE3) were grown at 37°C in 1 L of Lysogeny Broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) containing 50 μg/mL kanamycin or 100 μg/mL ampicillin. IPTG was added to a final concentration of 0.05 mM at mid-log phase and incubation continued at 30°C for 5 h. Cells were collected by centrifugation and re-suspended in 40 mL of 10 mM sodium phosphate buffer, pH 6.0. To the suspension, 100 U Benzonase nuclease (Novagen) and 400 mU lysozyme were added and incubated for 20 min at room temperature. The cells were subsequently passed through a pressure homogenizer three times. The cell lysate was clarified by centrifugation at 16,000*g* for 20 min at 4°C. The pellet of cell debris and insoluble protein (inclusion bodies) was saved for refolding experiments. The supernatant was passed through a 0.22 μm Millipore Millex® syringe filter and purified by binding to a 5 mL HiTrap SP-FF sepharose cation exchange column (GE Healthcare). After washing with 50 mL of 10 mM sodium phosphate buffer, pH 6.0, the protein was eluted with a sodium chloride gradient (10 mM sodium phosphate, pH 6.0, 0–500 mM sodium chloride, 50 mL) using an ÄKTA purifier. Fractions (30 × 5 mL) were collected and protein elution was monitored by absorption at 280 nm. The purity of the desired fractions was

checked by SDS-PAGE. Fractions were combined and concentrated using a 10 kDa MWCO spin filter (Millipore) and the buffer was exchanged to 10 mM sodium phosphate (pH 6.0) using the same spin filter.

Refolding screen

Lyophilized native wild-type Bcx (400 μg) was dissolved in 400 μL denaturing buffer (sodium phosphate 50 mM, pH 6.0 containing 6 M GuHCl, final protein concentration 1 mg/mL) and denatured by incubation for at least 2 hr at room temperature. Afterwards, protein concentration was determined by ultraviolet absorbance at 280 nm (predicted $\varepsilon_{280} = 82{,}850\ M^{-1}\ cm^{-1}$). The fractional factorial layout was calculated using the package F2Fr in R[21] and is summarized in Tables SI and SII. In a 96-well plate, duplicates of the 32 refolding buffers (100 μL each) were prepared and cooled to 4°C. Denatured Bcx (5 μL) was diluted into each of the refolding buffers to yield a final concentration of 50 μg/mL and the plate was incubated overnight at 4°C. Turbidity of the solution was determined by measuring absorbance at 340 nm, 405 nm and 560 nm with a Synergy H1 Hybrid Reader (BioTek Instruments). Positive and negative controls were performed with the wells containing native Bcx and aggregated Bcx (unfolded protein diluted into buffer solutions containing no further additives), respectively. From the results of the screens, relative aggregation values were calculated as follows. The lowest absorption value in the screen was subtracted from all values to set the positive controls (50 μg/mL native Bcx in buffer) to 0 aggregation. The differences where then divided by the highest absorption value (denatured Bcx diluted at 50 μg/mL into buffer only, pH 3.5) and multiplied by 100 to obtain the relative aggregation values normalized to highest aggregation in the screen (100%). From the normalized data, statistical plots for further analysis were compiled with the respective packages in R.

Activity assay for Bcx

Enzymatic activity was assayed according to established protocols.[7] An end-point assay (substrate concentration $>5 \times K_M$) was performed for each well containing low aggregation in duplicates using 5 mM 2,5-DNP β-xylobioside as a substrate in assay buffer (20 mM MES pH 6.0, 50 mM sodium chloride, 0.1% BSA). Refolded Bcx (0.7 μL) from the wells was directly diluted into 70 μL assay buffer at 20°C and the reaction was monitored by measuring absorption of the released 2,5-dinitrophenolate at 440 nm ($\varepsilon_{440} = 3.57\ mM^{-1}\ cm^{-1}$). Under these conditions with substrate at a saturating concentration, the initial reaction velocity was taken to provide a relative measure of the amount of active enzyme present in a given sample. Relative activity was calculated as the percentage of this velocity versus the positive control, which contained the same amount of native Bcx added to the refolding buffer of each well (5.0 μg in 100 μL). Relative activity was calculated with reference to the value of native Bcx (positive control) at 20°C.

Refolding milligram quantities of Bcx by pulsed dilution

Lyophilized wild-type Bcx (10 mg) was dissolved at room temperature in 10 mL of denaturing buffer (7 M GuHCl, 50 mM sodium phosphate, pH 6.0) to a final protein concentration of 1 mg/mL (50 μM) for at least 2 h. The protein solution was then added dropwise to 200 mL of refolding buffer (4°C) at a rate of ~1 drop every 5–10 s using a peristaltic pump. The refolding buffer consisted of 50 mM HEPES pH 7.0, 0.06% w/v PEG 3350, 0.5 mM Tween-80, 1.0 M GuHCl. After addition was complete, the refolded protein was incubated overnight at 4°C. Precipitated protein was removed by centrifugation (31,000*g*, 30 min). The refolded protein was exchanged into 10 mM phosphate buffer pH 6.0 with an Amicon® Stirred Cell (membrane MWCO of 10,000 Da) at 4°C, and subsequently concentrated to a volume of 40 mL. Refolded Bcx was purified by cation exchange chromatography to remove PEG 3350 (ÄKTA Purifier equipped with HiTrap SP-FF (GE Healthcare), 10 mM sodium phosphate pH 6.0, 0–250 mM NaCl in 20 min).

Refolding protein from inclusion bodies

The pellet obtained from the insoluble fraction was washed twice with 10 mM sodium phosphate buffer pH 6.0 containing 1% w/v Triton X-100 (Fisher, electrophoresis grade), once with buffer containing 1 M sodium chloride and once with water. The inclusion bodies were dissolved by stirring at room temperature in 10 mL of denaturing buffer (7 M GuHCl, 50 mM sodium phosphate, pH 6.0, and 1 mM DTT for cysteine-containing mutants, final protein concentration 1 mg/mL). Fast mixing of the protein into the denaturing buffer and dissolution of the inclusion bodies within a few minutes was found to be crucial to avoid aggregation of unfolded protein and low refolding yields. The solution of denatured protein was filtered through a 0.22 μm syringe filter (Millipore) or centrifuged (31,000*g*, 30 min) to remove any undissolved protein. Refolding by pulsed dilution was accomplished by the same protocol for native Bcx described above.

NMR spectroscopy

The ^{15}N-HSQC spectra of ^{15}N-ced Bcx were recorded at 25°C with a cryoprobe-equipped 600 MHz Bruker Avance III spectrometer, and processed using Bruker Topspin 3.1 The samples contained 0.2 mM protein

in 10 mM phosphate buffer pH 6.0 with 5% D_2O lock solvent.

Acknowledgments

We thank Dr. Hong-Ming Chen for the synthesis of the DNP xylobioside used in kinetic analyses and Dr. Lars Baumann for helpful discussions.

Conflict of Interest

The authors have no conflicts of interest.

References

1. Viikari L, Kantelinen A, Sundquist J, Linko M (1994) Xylanases in bleaching: from an idea to the industry. FEMS Microbiol Rev 13:335–350.
2. Paës G, Berrin J-G, Beaugrand J (2012) GH11 xylanases: structure/function/properties relationships and applications. Biotechnol Adv 30:564–592.
3. Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326–338.
4. Juturu V, Wu JC (2012) Microbial xylanases: engineering, production and industrial applications. Biotechnol Adv 30:1219–1227.
5. Wakarchuk WW, Campbell RL, Sung WL, Davoodi J, Yaguchi M (1994) Mutational and crystallographic analyses of the active site residues of the *Bacillus circulans* xylanase. Protein Sci 3:467–475.
6. Davoodi J, Wakarchuk WW, Campbell RL, Carey PR, Surewicz WK (1995) Abnormally high pK_a of an active-site glutamic acid residue in *Bacillus circulans* xylanase. Eur J Biochem 232:839–843.
7. Lawson SL, Wakarchuk WW, Withers SG (1996) Effects of both shortening and lengthening the active site nucleophile of *Bacillus circulans* xylanase on catalytic activity. Biochemistry 35:10110–10118.
8. McIntosh LP, Hand G, Johnson PE, Joshi MD, Körner M, Plesniak LA, Ziser L, Wakarchuk WW, Withers SG (1996) The pK_a of the general acid/base carboxyl group of a glycosidase cycles during catalysis: a ^{13}C-NMR study of *Bacillus circulans* xylanase. Biochemistry 35:9958–9966.
9. Plesniak LA, Connelly GP, Wakarchuk WW, McIntosh LP (1996) Characterization of a buried neutral histidine residue in *Bacillus circulans* xylanase: NMR assignments, pH titration, and hydrogen exchange. Protein Sci 5:2319–2328.
10. Plesniak LA, Wakarchuk WW, McIntosh LP (1996) Secondary structure and NMR assignments of *Bacillus circulans* xylanase. Protein Sci 5:1118–1135.
11. Joshi MD, Hedberg A, McIntosh LP (1997) Complete measurement of the pK_a values of the carboxyl and imidazole groups in *Bacillus circulans* xylanase. Protein Sci 6:2667–2670.
12. Lawson SL, Wakarchuk WW, Withers SG (1997) Positioning the acid/base catalyst in a glycosidase: studies with *Bacillus circulans* xylanase. Biochemistry 36: 2257–2265.
13. Zechel DL, Withers SG (1999) Glycosidase mechanisms: anatomy of a finely tuned catalyst. Acc Chem Res 33:11–18.
14. Sung WL, Luk CK, Zahab DM, Wakarchuk W (1993) Overexpression of the *Bacillus subtilis* and *circulans* xylanases in *Escherichia coli*. Prot Express Purif 4: 200–206.
15. Hahn M, Piotukh K, Borriss R, Heinemann U (1994) Native-like *in vivo* folding of a circularly permuted jellyroll protein shown by crystal structure analysis. Proc Natl Acad Sci USA 91:10417–10421.
16. Plaxco KW, Spitzfaden C, Campbell ID, Dobson CM (1996) Rapid refolding of a proline-rich all-beta-sheet fibronectin type III module. Proc Natl Acad Sci USA 93:10703–10706.
17. Jahn TR, Parker MJ, Homans SW, Radford SE (2006) Amyloid formation under physiological conditions proceeds via a native-like folding intermediate. Nat Struct Mol Biol 13:195–201.
18. Willis MS, Hogan JK, Prabhakar P, Liu X, Tsai K, Wei Y, Fox T (2005) Investigation of protein refolding using a fractional factorial screen: a study of reagent effects and interactions. Protein Sci 14:1818–1826.
19. Tsumoto K, Ejima D, Kumagai I, Arakawa T (2003) Practical considerations in refolding proteins from inclusion bodies. Protein Expr Purif 28:1–8.
20. Reitinger S, Yu Y, Wicki J, Ludwiczek M, D'Angelo I, Baturin S, Okon M, Strynadka NCJ, Lutz S, Withers SG, McIntosh LP (2010) Circular permutation of *Bacillus circulans* xylanase: a kinetic and structural study. Biochemistry 49:2464–2474.
21. R: A language and environment for statistical computing, R Foundation for Statistical Computing (2013), Vienna, Austria, http://www.R-project.org

A3	A7	17	Citrate pH 3.5	Tween-80	0	0	0	0	0	1	1	0.27	48%	
B3	B7	18	Acetate pH 4.6	Tween-80	1	0	1	0	0	1	0	0.22	17%	
C3	C7	19	MES pH 5.5	Tween-80	0	1	0	1	0	1	1	0.19	4%	+
D3	D7	20	MES pH 6.5	Tween-80	0	1	1	0	0	1	0	0.20	6%	++
E3	E7	21	HEPES pH 7.0	Tween-80	0	0	1	0	0	0	1	0.20	9%	+
F3	F7	22	PBS pH 7.5	Tween-80	0	0	0	1	0	0	0	0.21	15%	
G3	G7	23	TRIS pH 8.5	Tween-80	0	0	1	0	1	0	0	0.24	30%	
H3	H7	24	Borate pH 9.5	Tween-80	1	1	0	1	1	0	1	0.24	30%	
A4	A8	25	Citrate pH 3.5	NDSB 201	0	0	0	0	1	1	0	0.22	22%	
B4	B8	26	Acetate pH 4.6	NDSB 201	1	1	1	1	0	1	0	0.20	10%	+
C4	C8	27	MES pH 5.5	NDSB 201	1	1	0	1	0	0	0	0.21	13%	+
D4	D8	28	MES pH 6.5	NDSB 201	0	0	1	1	0	1	0	0.21	15%	
E4	E8	29	HEPES pH 7.0	NDSB 201	0	1	1	1	0	0	1	0.22	22%	
F4	F8	30	PBS pH 7.5	NDSB 201	1	1	0	0	0	1	1	0.20	6%	+
G4	G8	31	TRIS pH 8.5	NDSB 201	1	1	1	1	1	1	1	0.24	32%	
H4	H8	32	Borate pH 9.5	NDSB 201	1	0	0	1	0	1	1	0.22	18%	

Denatured Bcx diluted into buffer without any additives (negative controls)													
	A9		Citrate pH 3.5	0	0	0	0	0	0	0	0	0.32	78%
	B9		Acetate pH 4.6	0	0	0	0	0	0	0	0	0.36	100%
	C9		MES pH 5.5	0	0	0	0	0	0	0	0	0.30	64%
	D9		MES pH 6.5	0	0	0	0	0	0	0	0	0.25	36%
	E9		HEPES pH 7.0	0	0	0	0	0	0	0	0	0.24	28%
	F9		PBS pH 7.5	0	0	0	0	0	0	0	0	0.25	35%
	G9		TRIS pH 8.5	0	0	0	0	0	0	0	0	0.22	20%
	H9		Borate pH 9.5	0	0	0	0	0	0	0	0	0.23	27%
Native Bcx diluted in buffer without any additives (positive controls)													
	A10		Citrate pH 3.5	0	0	0	0	0	0	0	0	0.19	3%
	B10		Acetate pH 4.6	0	0	0	0	0	0	0	0	0.19	4%
	C10		MES pH 5.5	0	0	0	0	0	0	0	0	0.19	5%
	D10		MES pH 6.5	0	0	0	0	0	0	0	0	0.18	0%
	E10		HEPES pH 7.0	0	0	0	0	0	0	0	0	0.22	20%
	F10		PBS pH 7.5	0	0	0	0	0	0	0	0	0.19	1%
	G10		TRIS pH 8.5	0	0	0	0	0	0	0	0	0.19	2%
	H10		Borate pH 9.5	0	0	0	0	0	0	0	0	0.20	7%

Supporting Information Table SII. Sixteen condition fractional factorial screen for refinement. Each condition contained 0.06% w/v PEG 3350 except the controls. Factors: detergent (0 = 1.0 M NDSB-201; 1 = 0.50 mM Tween-20); GuHCl concentration (0 = 0.5 M GuHCl; 1 = 1.0 M GuHCl), sucrose (0 = no sucrose; 1 = 440 mM sucrose); arginine (0 = no arginine HCl; 1 = 550 mM arginine). Relative aggregation values were calculated according to the Materials and Methods section. Relative activity values were calculated with reference to the positive control in position E5. The color coding is a visual guide.

Position	Run Order	Buffer (50 mM)	Detergent	GuHCl	Arginine	Sucrose	A340 mean	Rel. aggregation	v_0 (A/min)	v_0 (M/s)	Rel. activity
A1	1	MES	NDSB	0	1	0	0.227	52%			
B1	2	MES	Tween	1	1	1	0.213	37%			
C1	3	HEPE	Tween	1	0	1	0.194	17%	0.11	5.0 x 10^{-}	68%
D1	4	TRIS	Tween	1	1	0	0.216	40%			
E1	5	MES	Tween	1	0	1	0.206	29%			
F1	6	MES	NDSB	1	0	1	0.209	33%			
G1	7	HEPE	Tween	0	1	0	0.216	40%			
H1	8	TRIS	Tween	0	0	0	0.193	16%	0.11	5.0 x 10^{-}	68%
A2	9	MES	NDSB	1	0	1	0.207	31%			
B2	10	MES	NDSB	1	1	0	0.219	43%			
C2	11	HEPE	Tween	1	1	0	0.209	33%			
D2	12	TRIS	NDSB	0	0	0	0.191	14%	0.086	4.0 x 10^{-}	54%
E2	13	MES	NDSB	0	0	1	0.192	15%	0.090	4.2 x 10^{-}	57%
F2	14	MES	Tween	0	0	0	0.212	36%			
G2	15	HEPE	Tween	1	0	0	0.183	5%	0.13	6.1 x 10^{-}	82%
H2	16	TRIS	NDSB	1	0	0	0.191	14%	0.081	3.8 x 10^{-}	51%
A3	17	MES	NDSB	1	0	0	0.192	15%			

B3	18	MES	NDSB	0	1	1	0.224	48%			
C3	19	HEPE	Tween	1	0	0	0.187	9%	0.12	5.7 x	77%
D3	20	TRIS	Tween	1	1	1	0.189	12%	0.064	3.0 x	40%
E3	21	MES	Tween	0	1	1	0.218	42%			
F3	22	MES	NDSB	0	1	1	0.214	38%			
G3	23	HEPE	Tween	0	1	0	0.218	42%			
H3	24	TRIS	Tween	0	1	1	0.194	17%			
A4	25	MES	NDSB	0	0	0	0.199	22%			
B4	26	MES	NDSB	1	1	0	0.208	32%			
C4	27	HEPE	NDSB	0	1	0	0.206	29%			
D4	28	TRIS	Tween	0	0	1	0.201	24%			
E4	29	MES	NDSB	1	1	1	0.207	31%			
F4	30	MES	Tween	0	0	1	0.191	14%	0.078	3.6 x	49%
G4	31	HEPE	NDSB	0	0	1	0.204	27%			
H4	32	TRIS	NDSB	1	1	1	0.221	45%			

Denatured Bcx diluted into buffer without any additives (negative controls)											
A5		MES pH 5.5	-	-	0	0	0.273	100%	0.005		0.3%
B5		MES pH 6.5	-	-	0	0	0.239	64%			
C5		HEPES pH 7.0	-	-	0	0	0.225	49%			
D5		TRIS 8.5	-	-	0	0	0.222	46%			
Native Bcx diluted in buffer without any additives (positive controls)											
E5		MES pH 5.5	-	-	0	0	0.178	0%	0.23		100%
F5		MES pH 6.5	-	-	0	0	0.184	6%			
G5		HEPES pH 7.0	-	-	0	0	0.187	9%			
H5		TRIS pH 8.5	-	-	0	0	0.195	18%			

Supporting Information Table SIII. Sequences of Bcx permutants.

Permutant /Mutant		Sequence
Wild-type	Protein	ASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSNTGNFVVGKGWTTGSPFRTI NYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTGTYKGTVKSDGG TYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNAWKSHG MNLGSNWAYQVMATEGYQSSGSSNVTVW
	DNA	ATGGCTAGCACAGATTACTGGCAAAACTGGACAGACGGTGGCGGTATCGTT AATGCCGTGAACGGCTCCGGAGGCAACTACAGCGTGAATTGGTCTAATACT GGGAACTTCGTAGTCGGAAAAGGTTGGACGACAGGATCCCCGTTCCGTACG ATCAACTACAACGCTGGCGTTTGGGCCCCGAATGGTAACGGTTACCTGACA CTGTATGGCTGGACGCGTTCGCCACTGATTGAATATTACGTTGTCGACTCTT GGGGAACGTACCGTCCGACTGGAACCTACAAAGGCACAGTCAAAAGCGAT GGTGGTACCTATGACATCTACACCACCACAAGATACAACGCACCTTCCATCG ATGGCGATCGGACCACCTTTACTCAGTATTGGAGTGTTAGACAATCTAAGCG GCCGACTGGTTCGAACGCCACCATTACGTTCACCAATCACGTGAATGCATG GAAATCCCACGGTATGAACCTAGGTTCTAATTGGGCTTATCAAGTAATGGCG ACCGAAGGCTACCAGAGCTCTGGTTCTTCCAACGTTACAGTGTGGTAA
cpP75-T87C	Protein	PLIEYYVVDSWGCYRPTGTYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQY WSVRQSKRPTGSNATITFTNHVNAWKSHGMNLGSNWAYQVMATEGYQSSGS SNVTVWGGASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSNTGNFVVGKGW TTGSPFRTINYNAGVWAPNGNGYLTLYGWT
	DNA	ATGCGTTCGCCACTGATTGAATATTACGTTGTCGACTCCTGGGGATGCTACC GTCCGACTGGAACCTACAAAGGCACAGTCAAAAGCGATGGTGGTACCTATG ACATCTACACCACCACAAGATACAACGCACCTTCCATCGATGGCGATCGGA CCACCTTTACTCAGTATTGGAGTGTTAGACAATCTAAGCGGCCGACTGGTTC GAACGCCACCATTACGTTCACCAATCACGTGAATGCATGGAAATCCCACGG TATGAACCTAGGTTCTAATTGGGCTTATCAAGTAATGGCGACCGAAGGCTAC CAGAGCTCTGGTTCTTCCAACGTTACAGTGTGGGGCGGTGCTAGCACAGAT TACTGGCAAAACTGGACAGACGGTGGCGGTATCGTTAATGCCGTGAACGGC TCCGGAGGCAACTACAGCGTGAATTGGTCTAATACTGGGAACTTCGTAGTC GGAAAAGGTTGGACGACAGGATCCCCGTTCCGTACGATCAACTACAACGCT GGCGTTTGGGCCCCGAATGGTAACGGTTACCTGACACTGTATGGCTGGACG TAΩ
cpY174-S162C	Protein	YQSSGSSNVTVWGGASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSNTGNF VVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTY RPTGTYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTAYWSVRQSKRPTGSN ATITFTNHVNAWKSHGMNLGCNWAYQVMATEG
	DNA	ATGTATCAAAGCTCTGGCTCTTCCAACGTTACCGTGTGGGGCGGTGCTAGC ACAGATTACTGGCAAAACTGGACAGACGGTGGCGGTATCGTTAATGCCGTG AACGGCTCCGGAGGCAACTACAGCGTGAATTGGTCTAATACTGGGAACTTC GTAGTCGGAAAAGGTTGGACGACAGGATCCCCGTTCCGTACGATCAACTAC AACGCTGGCGTTTGGGCCCCGAATGGTAACGGTTACCTGACACTGTATGGC TGGACGCGTTCGCCACTGATTGAATATTACGTTGTCGACTCTTGGGGAACG TACCGTCCGACTGGAACCTACAAAGGCACAGTCAAAAGCGATGGTGGTACC TATGACATCTACACCACCACAAGATACAACGCACCTTCCATCGATGGCGATC GGACCACCTTTACTCAGTATTGGAGTGTTAGACAATCTAAGCGGCCGACTG GTTCGAACGCCACCATTACGTTCACCAATCACGTGAATGCATGGAAATCCCA CGGTATGAACCTAGGTTGCAATTGGGCTTATCAAGTAATGGCGACCGAAGG CTAA