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Oxidative Folding of Reduced and Denatured Huwentoxin-I

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Huwentoxin-I, a neurotoxic peptide with 33 amino acid residues and three disulfide bonds, was used to investigate the pathway of reduction/denaturation and of oxidative folding in small proteins with multiple disulfide bonds. Titration of thiol groups, reversed-phase HPLC, 1D NMR spectroscopy, and biological activity assays were used to monitor the extent of reduction/denaturation and renaturation of the toxin. The reduction and denaturation of huwentoxin-I resulted in a 100% loss of bioactivity as measured in a mouse phrenic nerve-diaphragm preparation. About 90% of full biological activity could be restored under optimized conditions of oxidative refolding of the reduced peptide. Several reaction conditions employing air oxidation, oxidized and reduced glutathione (GSSG and GSH), and cystine/cysteine were investigated in order to find optimal conditions for renaturation of huwentoxin-I. The best renaturation yield was achieved in 0.1 mM GSSG and 1 mM GSH at pH 8.5 and 4°C over 24 hr. High concentrations of glutathione and high temperatures reduced renaturation yields. Oxidative refolding of huwentoxin-I in air requires about 6 days for maximal yields and is inhibited by EDTA.

KEY WORDS: Huwentoxin-I; spider toxin; disulfide bond; denaturation; renaturation.

1. INTRODUCTION

The folding of proteins with concomitant disulfide bond formation entails both the establishment of native, noncovalent interactions and the formation of covalent bonds. In the case of proteins with multiple disulfide bonds, the number of possible combinations increases rapidly. For example, there are 15 and 105 possible ways to form three and four intramolecular disulfide bonds, respectively. The mechanisms and the exact pathways for the correct folding and disulfide bond pairing of these proteins are still not clear, but optimization of conditions for the correct refolding and disulfide pairing of linear polypeptides is possible. Recently a family of small proteins (M_r <5000) containing three or four disulfide bonds, e.g., ω-conotoxin (Pallaghy *et al.*, 1993), *C. maxima* trypsin inhibitor I (Holak *et al.*, 1991), gurmarin (Imoto *et al.*, 1991) and huwentoxin-I (Qu *et al.*, 1997), were found to have structural similarity, but functional diversity. They share a structure referred to as the inhibitor cystine knot motif (Pallaghy *et al.*, 1994). To investigate the relationship of the structure and function of these small proteins it is necessary to carry out chemical peptide synthesis or gene expression to obtain the native proteins or their analogues. Very often the synthetic and recombinant products have low biological activity due to improper folding and disulfide bridging. Therefore, the investigation of the pathways and conditions for folding of this family of proteins has both theoretical and practical significance.

Huwentoxin-I is a neurotoxin isolated from the venom of the Chinese bird spider *Selenocosmia huwena.* It contains 33 amino acid residues and three disulfide bonds with the linkages, Cys2 to Dys16, Cys9 to Cys22, and Cys17 to Cys29 (Liang *et al.* 1993*a;* Zhang and Liang, 1993). The three-dimensional structure of this toxin has been determined by 2D NMR.[3] The structure

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[3] Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; NMR, nuclear magnetic resonance.

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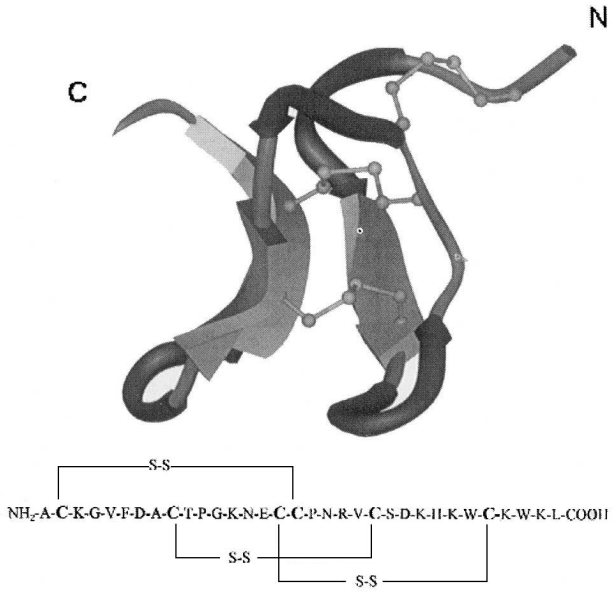

Fig. 1. The backbone folding structure (top) and amino acid sequence and disulfide arrangement (bottom) of huwentoxin-I.

has a typical inhibitor cystine knot motif (Qu *et al.*, 1995, 1997) (Fig. 1). In this paper we report the results of reduction/denaturation and renaturation/oxidation studies on huwentoxin-I, as well as the optimization of the conditions for the oxidative refolding and disulfide bond pairing of this small protein.

2. EXPERIMENTAL

2.1. Materials

Native huwentoxin-I was purified according to the procedure described by Liang *et al.* (1993). Reduced and oxidized glutathione (GSH and GSSG, respectively), dithiothreitol (DTT), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and trifluoroacetic acid (TFA) were obtained from Sigma. All other reagents were of analytical grade.

2.2. Reduction and Denaturation of Huwentoxin-I

The procedure of Jaenicke and Rudolph (1990) was adapted for the reduction and denaturation of huwentoxin-I. Native huwentoxin-I was dissolved at a protein concentration of 1 mM in 0.1 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride, 0.1 M DTT, and 1 mM EDTA. The buffer was freshly prepared and was degassed by sonication under nitrogen. The sample solution was incubated at 25°C for 2 hr under nitrogen. After reduction, the solution was adjusted to pH 3 by dropwise addition of glacial acetic acid. The reduced toxin was separated from the denaturing and reducing agents by HPLC on a short (3.9 × 100 mm) C18 column. After the sample solution had been applied to the column, helium-saturated 0.1% TFA (in water) was pumped through to wash out the salts. The reduced toxin was eluted using helium-saturated 35% acetonitrile containing 0.1% TFA. The reduced huwentoxin-I was used immediately or was lyophilized and stored under nitrogen at −20°C.

2.3. Titration of Thiol Groups (Anderson and Wetlaufer, 1975)

Native or reduced huwentoxin-I (20 nmol) was dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.3) containing 6 M guanidinium chloride and 1 mM EDTA. The difference in absorbance at 412 nm between the toxin solution and a water reference solution was measured. Then 100 μl of 3 mM DTNB in 0.1 M phosphate buffer (pH 7.3) was added to the sample and reference solutions, and the resulting solutions were mixed thoroughly. The reactions were monitored at 412 nm and the differences in absorbance between the samples and reference were measured when the absorbance stopped increasing. The concentration of thiols was calculated using an extinction coefficient of 412 nm of 13,700 M^{-1} cm^{-1}.

2.4. Protein NMR Analysis

Native or reduced huwentoxin-I was dissolved in 0.5 ml of 20 mmol/L phosphate buffer (90% H_2O/10% D_2O) to give a final peptide concentration of 4 mM. The 1D NMR analysis was performed at 500 MHz on a Bruker AM-500 NMR spectrometer equipped with an Aspect 3000 computer. The ^{1}H-NMR spectra were recorded at 27°C, and 36,000 data points were acquired (128 scans per increment). The spectral width was 6494 Hz (12.987 ppm). Solvent suppression was carried out by using the presaturation method.

2.5. Assay of Biological Activity

Biological activity measurements were carried out using mouse phrenic nerve-diaphragm preparations. After dissection, the preparation was placed in a small Plexiglas chamber and immersed in Tyrode's solution alone or Tyrode's solution containing peptide, into

which was bubbled 95% O_2, 5% CO_2. Assays were conducted at 30–32°C. Electrical stimulation was applied indirectly to the phrenic nerve with a suction electrode, or directly to the muscle at a frequency of 0.2 Hz (supramaximal, 0.2 msec, square wave). The twitch responses were transformed into an electric signal by an electromechanical transducer made from a semiconductor strain gauge. The signals were amplified and recorded with a pen recorder.

2.6. Oxidative Folding of Denatured Huwentoxin-I by Air

The reduced and denatured huwentoxin-I was dissolved in 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 M guanidine hydrochloride at a concentration of 0.1 mg/ml. The solution was stirred in an open beaker exposed to air at 4°C for several days. At 2, 4, 8, 12, 48 hr and 3, 4, and 5 days a small amount of sample was removed and analyzed by HPLC to monitor the course of renaturation.

2.7. Oxidative Folding of Denatured Huwentoxin-I in the Presence of Cystine/Cysteine

The refolding buffer consisted of a freshly prepared solution of 0.1 mM cystine and 1 mM cysteine dissolved in 0.1 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA and 0.5 M guanidine hydrochloride. The reduced and denatured huwentoxin-I was dissolved in the buffer at a concentration of 0.1 mg/ml. Oxidative refolding was carried out by slowly stirring the solution at 4°C for 24 hr. After 2, 4, 8, 12, and 24 hr small samples of the mixture were analyzed by HPLC to determine the extent of renaturation.

2.8. Oxidative Folding of Denatured Huwentoxin-I in the Presence of GSSG/GSH

The reaction was carried out under the same conditions as above except that cysteine and cystine were replaced by reduced and oxidized glutathione, respectively. Three ratios of GSSG/GSH were tested: 0.1 mM/1 mM, 0.5 mM/5 mM, and 1 mM/10 mM.

2.9. HPLC Analysis of Native, Denatured, and Renatured Huwentoxin-I

Analysis of peptides was performed on a Waters Model 600E HPLC system equipped with a Model 996 photodiode array detector. The peptide samples were subjected to reversed-phase HPLC on a YWG-Pak C18 column (0.39 cm × 30 cm). Elution was performed with a linear gradient of acetonitrile into water containing 0.1% TFA to about 30–35% acetonitrile over 60 min at a flow rate of 0.6 ml/min at 45°C.

3. RESULTS AND DISCUSSION

The reduction/denaturation and renaturation/oxidation reactions of huwentoxin-I were monitored in four ways: titration of thiol groups, reversed-phase HPLC, 1D NMR analysis, and biological activity assay. Titration with Ellman's reagent of 10 nmol of purified reduced, denatured huwentoxin-I, prepared as described in Section 2, showed the liberation of 51 nmol of nitrothiobenzoate ion, compared with the expected 60 nmol if all six Cys residues were in the reduced form. The control experiment with 10 nmol of native huwentexin-I showed release of only 2.1 nmol of the expected 20 if one pair of the six Cys residues was in the reduced form. The 2.1 nmol of thiol detected is within the experimental error of the method. These results indicate that reduction of disulfide bonds and presumably unfolding of the protein were essentially complete.

Guanidine hydrochloride (0.5 M) was included in the refolding solutions of all of the oxidative refolding experiments with the aim of (1) increasing the solubility of the denatured peptide and (2) improving the yield of refolded peptide, as it has been widely reported that the addition of guanidine hydrochloride or urea can improve refolding yields. For example, the renaturation of reduced chymotrypsinogen was strongly enhanced by adding 1.0 M guanidine hydrochloride (Orsini and Goldberg, 1978). In the analysis of the oxidative folding, the HPLC column was operated at 45°C because the relatively high temperature improved resolution and reduced backpressure. It has been shown that huwentoxin-I has high thermal stability (Liang *et al.*, 1993*b*).

Figure 2 shows the reversed-phase HPLC chromatograms of the native, denatured, and air-oxidized/renatured huwentoxin-I at different reaction times. Figure 2B indicates that completely reduced and denatured huwentoxin-I has a longer retention time, probably due to the increased exposure of hydrophobic residues to solvent. Figure 2B also shows that the peak shape of the reduced and denatured huwentoxin-I is not as sharp as that of the native toxin, suggesting that it is a mixture of conformers with slightly different retention times. Figures 2C–E illustrate the retention

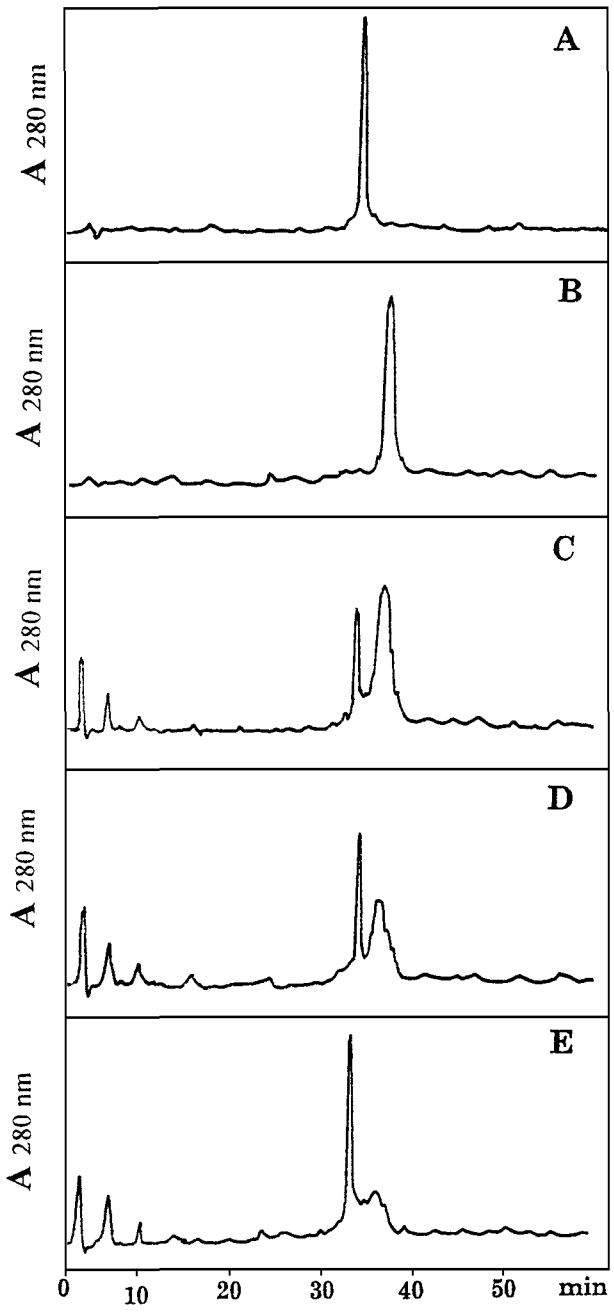

Fig. 2. Analytical reversed-phase HPLC chromatograms of (A) native huwentoxin-I; (B) reduced and denatured huwentoxin-I after desalting and purification; (C) reduced, denatured huwentoxin-I after air oxidation for 24 h; (D) after 3 days of air oxidation; (E) after 6 days (see Experiment Section 2 for HPLC conditions).

time changes that occur during reoxidation of reduced huwentoxin-I. The sharp peak in Figs. 2C–E has a retention time of 32.6 min, the same as that of the native toxin, and is therefore presumably the renatured three-disulfide bridged huwentoxin-I. After 1 day of air oxidation, the refolded product is about 31% of the total material (Fig. 2C). The yields of the refolded material increased to 53% and 81% on days 3 and 6, respectively. This particular oxidation reaction was stopped after 6 days, as no further increases in yield were observed. In our method of reoxidation, no EDTA was added to the reaction mixture, as other experiments (unpublished observations) indicated that the addition of EDTA gave very poor yields. It is believed (Ahmed *et al.*, 1975) that air oxidation is catalyzed by trace amounts of divalent metal ions, so chelating agents must be absent from the refolding solution. Figure 2 also demonstrates that the procedure of renaturation of huwentoxin-I can be monitored by reversed-phase HPLC.

The renatured huwentoxin-I, after purification by HPLC, showed 100% of the biological activity of the native toxin, as measured in the mouse phrenic nerve-diaphragm preparation (Fig. 3). On the other hand, the reduced and denatured huwentoxin-I, purified first to remove DTT and guanidine hydrochloride, was totally inactive (Fig. 3B).

Since we had previously deduced the sequence-specific ^{1}H-NMR assignments and the three-dimensional structure for native huwentoxin-1 (Qu *et al.*, 1995, 1997), we felt that comparison of the 1D NMR spectra of the denatured and renatured huwentoxin-I samples could give important structural insights. Figure 4 is a comparison of the 1D NMR spectra of the native, denatured, and renatured forms of huwentoxin-I. There are obvious differences between the native and denatured specimens; for example, the proton signals of the nine methyl groups from Ala1, Val5, Ala8, Val21, Thr10, and Leu33 in the region 0.0–2.0 ppm of the native toxin are completely shifted in the denatured sample and could not be identified. In the fingerprint region (6.0–9.0 ppm) the number of sharp proton peaks in denatured huwentoxin-I is significantly reduced and there are more broad peaks, indicating a mixture of conformations rather than a unique one. Significantly, the spectra of the renatured and native toxins are almost identical, indicating correct refolding upon renaturation.

The foregoing observations provide good evidence that the loss of biological activity on reduction of the disulfide bonds is due to unfolding and a large conformational change and that the recovery of activity is due to refolding to the native state.

Although the classical air oxidation of reduced, denatured huwentoxin-I gave promising results, the rate of reaction was very slow. Studies in other laboratories have demonstrated that an oxidative regeneration sys-

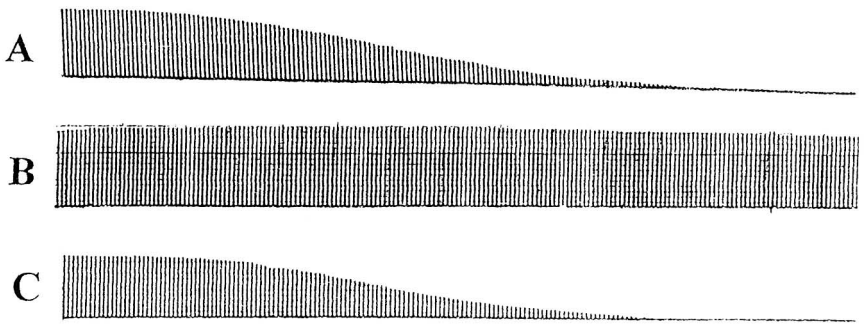

Fig. 3. The effect of 1×10^{-5} g/ml of toxins on neuromuscular transmission in the isolated mouse phrenic nerve-diaphragm preparation over 30 min: (A) native huwentoxin-I; (B) reduced and denatured huwentoxin-I; (C) reoxidized, renatured huwentoxin-I.

tem composed of low-molecular-weight disulfides and thiols can increase both the rate and yield of oxidation/renaturation by facilitating rapid reshuffling of incorrect disulfide bonds. The most extensively used disulfides and thiols are oxidized and reduced glutathione and cysteine/cystine. In order to find the best conditions for renaturation of reduced huwentoxin-I, we compared the oxidative refolding of huwentoxin-I by three methods: unfacilitated air oxidation, and air oxidation in the presence of GSSG/GSH or of cystine/cysteine.

Optimization of refolding under different conditions of buffer, time, redox reagent, etc., is very time-consuming, so only a limited number of conditions was investigated. Even so, this limited study provided much improved reaction conditions for the refolding of reduced huwentoxin-I. As shown in Fig. 5, the highest yield (89%) of renaturation was accomplished within 24 hr using 0.1 mM GSSG and 1 mM GSH. Figure 6 shows the analytical HPLC profiles for oxidation of reduced, denatured huwentoxin-I over 12 hr under different conditions. Simple air oxidation yielded several peaks after 12 hr (Fig. 6A). One peak, representing only 10% of the total material, was found to be similar to nat-

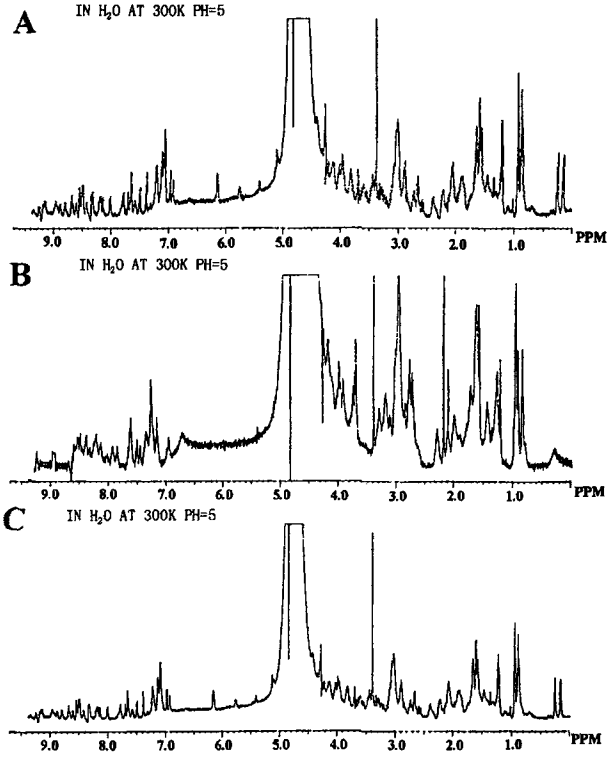

Fig. 4. One-dimensional ^{1}H-NMR spectra of (A) native huwentoxin-I, (B) reduced and denatured huwentoxin-I, (C) reoxidized, renatured huwentoxin-I.

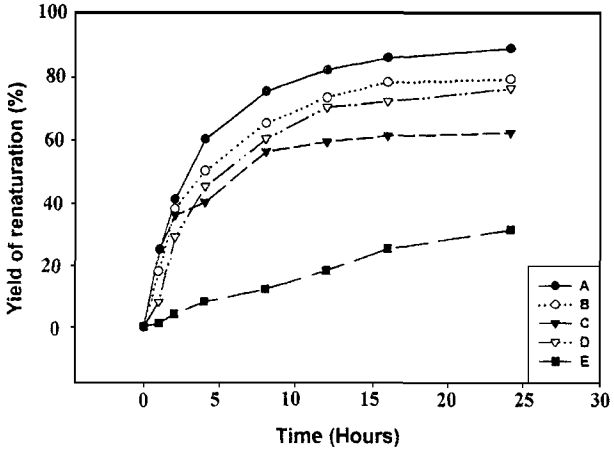

Fig. 5. Time course for oxidative refolding of reduced, denatured huwentoxin-I (A) in 0.1 mM GSSG and 1 mM GSH; (B) in 0.5 mM GSSG and 5 mM GSH; (C) in 1 mM GSSG and 10 mM GSH; (D) in 0.1 mM cystine and 1.0 mM cysteine; (E) under simple air oxidation.

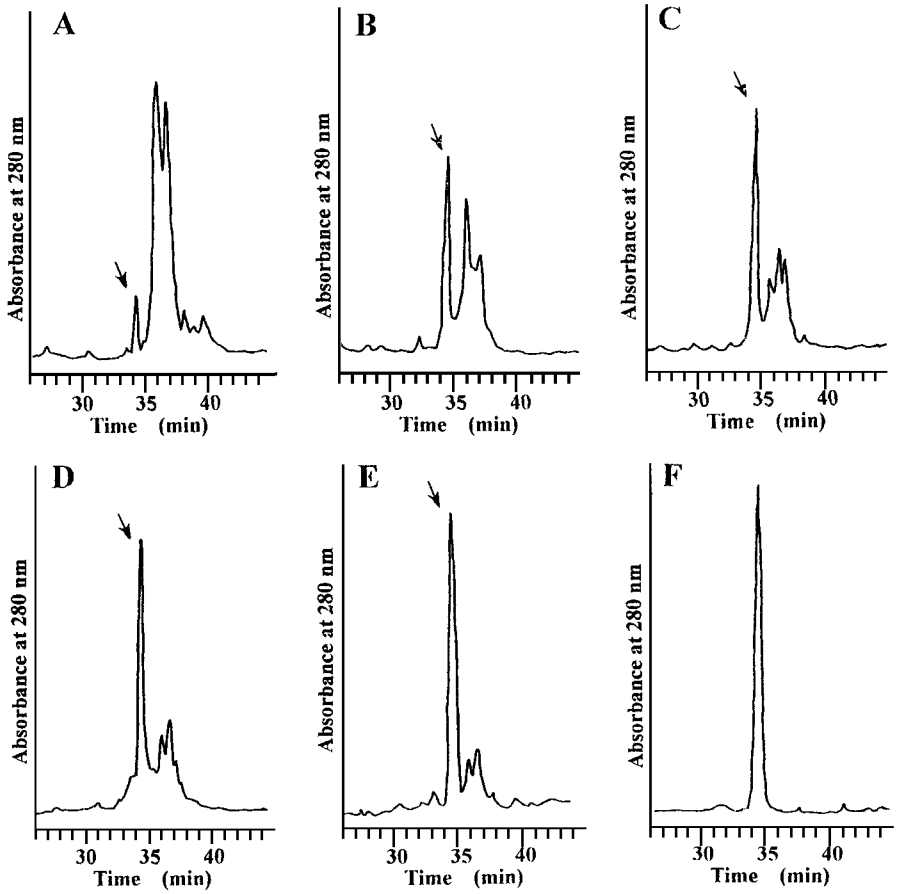

Fig. 6. Analytical reversed-phase HPLC of the oxidation products of reduced, denatured huwentoxin-I after 12 hr under various conditions: (A) air oxidation; (B) in 1 mM GSSG and 10 mM GSH; (C) in 0.1 mM cystine and 1.0 mM cysteine; (D) in 0.5 mM GSSG and 5 mM GSH; (E) in 0.1 mM GSSG and 1 mM GSH; (F) native huwentoxin-I.

ural huwentoxin-I in terms of HPLC retention time, absence of free thiol groups, and neurotoxic activity. The largest peak in Fig. 6A had about 25% huwentoxin-I activity, but appeared to be a mixture of molecules with different redox states which could not be easily separated. Oxidative folding in the presence of different concentrations of oxidized and reduced glutathione (Fig. 5B, D, E) resulted in the recovery of 50–80% of huwentoxin-I activity over 12 hr. The best result was obtained with GSSG/GSH 0.1 mM/1 mM (Fig. 6E); increasing the concentration of these reagents 5- to 10-fold gave successively lower yields. Because of the low solubility of cystine, only the buffer containing cystine/cysteine (0.1 mM/1 mM) was investigated. This reagent gave only 60% of huwentoxin-I activity in 12 hr. The lower performance of cystine/ cysteine is in contrast with the results of Lozanov *et al.* (1997), who reported higher yields of refolding of amaranth amylase inhibitor, a natural peptide with three disulfide bonds, in cystine/cysteine buffer than in glutathione buffers. As can be seen in Fig. 5, the yields of refolded huwentoxin-I are about the same in cystine/cysteine 0.1 mM/1 mM as in GSSG/GSH 0.5 mM/5 mM, but a little lower than those achieved under optimal (for this series of experiments) GSSG/GSH 0.1 mM/1 mM. It appears that no one set of refolding conditions is optimal for all proteins.

In summary, complete reduction and denaturation of huwentoxin-I caused a 100% loss of biological activity due to disulfide bond cleavage and conformational changes. About 90% of bioactivity activity could be restored under optimal refolding conditions, namely in the presence of GSSG/GSH (0.1 mM/1 mM) at pH 8.5 and 4°C. It was found that air oxidation and refolding were accelerated by both GSSG/GSH and cystine/cysteine, but that optimal concentrations had to be determined experimentally; for example, high concentrations of GSSG/GSH and high temperatures gave decreased yields of refolded product. Refolding in

the absence of a disulfide/thiol pair was very slow and was completely inhibited by EDTA.

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