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In Vitro Refolding of Porcine Pepsin Immobilized on Agarose Beads

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Since *in vitro* refolding of pepsin has long been attempted without success, it has been suspected that pepsin has no intrinsic refolding ability. In the present study, in order to eliminate unfavorable intermolecular interactions bringing about aggregation and autoproteolysis, we immobilized pepsin onto agarose beads. This technique enabled us to search extensively for appropriate refolding conditions without limitation of the refolding period. Renaturation of immobilized pepsin was observed exclusively at pH 3–5. This process was extremely slow and reached equilibrium after 300 h. Sixty percent of the proteolytic activity was recovered at pH 5. Addition of salts raised the recovery to 80% but had no significant effect on the refolding rate, suggesting that the salts mainly stabilize the native state of pepsin. This is the first report on the successful *in vitro* refolding of pepsin.

Key words: immobilization, multidomain protein, pepsin, pro-peptide, protein refolding.

Most extracellular proteases are synthesized with a pro-peptide, which is removed upon folding through an autolytic or exogenous proteolytic activity. These proteases can be classified into two from the point of view of *in vitro* refolding. One class, which includes chymotrypsin, trypsin, and elastase, readily refolds after chemical denaturation, even in the absence of the pro-peptides, as long as the disulfide bonds remain intact. The second class, represented by α-lytic protease and subtilisin *BPN'*, requires its own pro-peptide for efficient refolding (*1–3*). In the absence of pro-peptide, proteases of the second class are reported to fold to a molten globule-like intermediate state (*3*, *4*). Sohl *et al.* have shown that folding intermediate state of α-lytic protease is more stable than its native state (*5*). Thus, pro-peptide–dependent folding of proteases of the second class might call into question the validity of Anfinsen's dogma that the amino-acid sequence encodes all the information necessary for a protein to adopt its native three-dimensional structure (*6*). Given these circumstances, it is of vital importance to examine a possibility that a protease categorized in the second class has the potential to refold *in vitro* to an active state without the pro-peptide.

Porcine pepsin has been classified into the second class of protease since its *in vitro* refolding has not been accomplished in spite of extensive trials (*7–9*). Judgment about the refolding ability of a protein is often hindered by intermolecular interactions that bring about aggregation. In the case of protease refolding, it is possible that molecules already renatured attack their as yet denatured counterparts. The conditions used in the previous studies of pepsin refolding did not prevent aggregation and/or autoproteolysis completely. In addition, refolding conditions have been sought within the limits of a short refolding period. In the present study, in order to suppress intermolecular interactions unfavorable for refolding, we immobilized pepsin onto agarose beads. This technique, whose usefulness has been demonstrated in several studies of protein refolding (*10–13*), enabled us to search extensively for appropriate refolding conditions of pepsin without limitation of the refolding period. Here we report for the first time that pepsin is actually capable of refolding after chemical denaturation without the aid of the pro-peptide.

MATERIALS AND METHODS

Materials—Porcine pepsin (4,500 units/mg of protein) and bovine hemoglobin were purchased from Sigma. Cyanogen bromide (CNBr)–activated sepharose 4B was purchased from Amersham Pharmacia Biotech UK Ltd. All other reagents were of analytical grade.

Preparation of Immobilized Pepsin—The immobilized pepsin was prepared by the procedure described previously (*11*) with a slight modification. The CNBr-activated sepharose 4B gel was washed successively with 1 mM HCl and the coupling buffer (0.1 M sodium acetate buffer, pH 5.8, containing 0.5 M NaCl). Pepsin dissolved in 1 mM HCl was added to the gel suspension to give 0.1 mg of protein/ml of gel. The mixture was stirred gently at 4°C for 24 h, then washed with the coupling buffer to remove uncoupled molecules. Uncoupled groups on the resin were blocked by reaction with 0.2 M glycine, pH 5.0, at 4°C for 24 h. The filtered gel was washed successively with the coupling buffer, followed by 1 mM HCl containing 0.5 M NaCl. The washing procedure was repeated three times. The immobilized pepsin was stored in 1 mM HCl at 4°C.

Assay of Proteolytic Activity—Enzymatic activity of the immobilized pepsin was assayed based on proteolysis of acid-denatured hemoglobin. The gel was washed with 10 mM HCl prior to the assay. The reaction was started by the addition of 60 μl of 50% (v/v) gel suspension to 540 μl of acid-denatured hemoglobin solution (5 mg/ml in 10 mM

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Abbreviations CNBr, cyanogen bromide, GdnHCl, guanidine hydrochloride

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HCl), and the mixture was gently stirred in a test tube with a magnetic stirrer at 37°C At various intervals after mixing, the reaction was stopped by the addition of 600 μl of 10% (w/v) trichloroacetic acid solution. Samples were subjected to centrifugation and filtration to remove gels and the residual substrate. The pepsin activities were estimated from the increase in UV absorbance at 280 nm of the supernatants. The specific activity of the immobilized pepsin was 100 units/ml of gel.

Denaturation and Renaturation of Immobilized Pepsin—The immobilized pepsin was denatured in 0.1 M Tris-HCl buffer, pH 8.0, containing 8 M guanidine hydrochloride (GdnHCl) by gentle stirring for 3 h at 25°C It was confirmed that the denatured pepsin had lost its enzymatic activity completely. For renaturation, the agarose beads coupled with the denatured pepsin were filtered to remove GdnHCl and washed with 0.1 M citrate buffer (pH 2–3), 0 1 M phosphate buffer (pH 4–7), 0.1 M Tris-HCl buffer (pH 8), or 1 M lysine monohydrochloride (pH 6), then incubated in the same solution in a Falcon tube end over end at 25°C. At intervals, a portion of the gel suspension was taken from the tube, washed with 10 mM HCl, and subjected to the assay.

RESULTS AND DISCUSSION

Immobilized pepsin was denatured by incubation with 8 M GdnHCl, pH 8.0 at 25°C for 3 h, under which conditions it lost its enzymatic activity completely. Refolding of the immobilized pepsin was attempted under various conditions. Figure 1 shows the effect of pH on the time course of renaturation. Little or no renaturation occurred at pH 2, where pepsin exhibits optimum enzymatic activity. No renaturation was also observed under neutral or basic conditions (>pH 6). Marked enzymatic activity was recovered at pH 4–5. The maximum yield of renaturation was about 60% at pH 5. A striking feature of pepsin renaturation was that it proceeded extremely slowly, reaching equilibrium 300 h after initiating of the refolding.

Next, we attempted to optimize the solution conditions in order to achieve a higher refolding yield. Figure 2 shows the effects on renaturation of immobilized pepsin of addition of 1 M NaCl into the refolding solutions. Improvement of the refolding yields in the presence of 1 M NaCl was observed at pH 3–6, and a particularly marked improvement was achieved at pH 6. The addition of NaCl improved the final refolding yield but did not accelerate the refolding rate (Figs 1 and 2), suggesting that the salt affects thermodynamic equilibrium rather than the kinetics of refolding

An enhancing effect of salts has been also observed in the refolding of subtilisin *BPN'* (*11, 14*). However, in contrast to the pepsin refolding, the enhancement results mainly from an acceleration of the refolding rate: the pro-peptide of subtilisin stabilizes the transition state of the folding reaction and accelerates the folding rate by a factor of 10^3–10^5 (*2, 15*). Similarly, salts are supposed to stabilize the transition state of the refolding reaction of subtilisin *BPN'*. In the case of pepsin refolding, however, salts seem to stabilize the native state but not the transition state. Since pepsin is a highly acidic protein, it is conceivable that intramolecular electrostatic repulsion among acidic residues preclude the refolding We suggest that the salt weakens the electrostatic repulsion, rendering the native state of pepsin more stable.

The pro-peptide of pepsinogen is abundant in basic amino acid residues, *i.e.*, nine Lys and two Arg residues out of 44 amino acid residues. X-ray crystallographic study has indicated that the pro-peptide is accommodated just between the two domains of pepsin (*16*). One might expect that basic residues contained in the pro-peptide neutralize the electrostatic repulsion between the domains, stabilizing the folded state. The improvement of the refolding yield was further enhanced by use of L-lysine in place of NaCl: refolding yield was increased by approximately 45% at pH 6 (Fig. 3) The preference of lysine is probably due to its similarity to the lysine residues acting in the pro-peptide as an appropriate neutralizer.

Ahmad and McPhie have reported that pepsin could not reach to the native state even if the proteolytic activity was covalently inhibited and the refolding was performed at low pepsin concentration (*7*). They speculated that the failure is not due to aggregation but to an intrinsic property of the

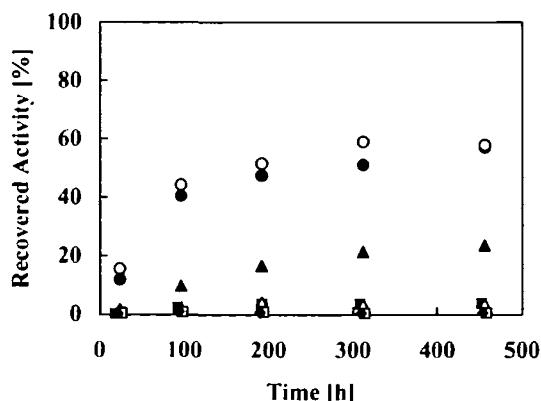

Fig 1 **Renaturation time course of immobilized pepsin.** Immobilized pepsin that had been unfolded in 8 M GdnHCl pH 8 0 at 25°C for 3 h was refolded by replacing the denaturation solution with 0.1 M citrate buffer (pH 2–3), 0 1 M phosphate buffer (pH 4–7), or 0 1 M Tris-HCl buffer (pH 8) The gel suspension was sampled at the times indicated and assayed for recovered pepsin activity ■, pH 2, ▲, pH 3, ●, pH 4, ○, pH 5; △, pH 6, □, pH 7, ◆, pH 8.

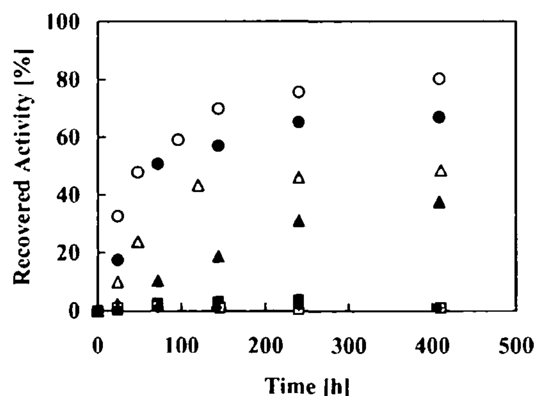

Fig. 2 **Effect of 1 M NaCl on the renaturation of immobilized pepsin.** Denatured immobilized pepsin was refolded at 25°C in the buffers of various pHs containing 1 M NaCl Recovered activity was measured at the times indicated. ■, pH 2, ▲, pH 3; ●, pH 4, ○, pH 5, △, pH 6; □, pH 7, ◆, pH 8

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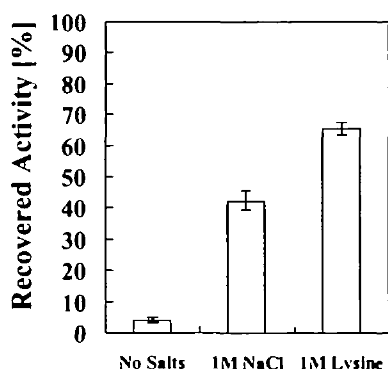

Fig 3 **Enhancement of renaturation of immobilized pepsin by lysine.** Denatured immobilized pepsin was refolded in 0 1 M phosphate buffer, pH 6 0, containing 1 M NaCl or 1 M lysine monohydrochloride, pH 6 0 Recovered activities were measured after 240 h of incubation at 25°C

pepsin molecule, whose active conformation is largely predetermined in the presence of the pro-peptide. The present study clearly shows that pepsin *per se* can fold to the native state without the aid of the pro-peptide if the pH is appropriate It is possible that the refolding of pepsin proceeded too slowly to detect within the experimental time-range of the previous studies using soluble pepsin.

The extremely slow refolding process of immobilized pepsin is in marked contrast with those of small monomeric proteins, which generally renature very rapidly. Slow refolding has been observed in *in vitro* refolding of firefly luciferase (*17*). It has been reported that reactivation of luciferase is attained after about 72 h, which has been shown to be irrelevant to proline isomerization (*17*). Since pepsin and firefly luciferase are both multidomain proteins, it could be speculated that refolding of individual domains proceeds independently and rapidly, while the process of adjustment of the interdomain packing between folded domains requires a much longer time. It is possible that further optimization of refolding conditions, *e.g.*, stepwise or gradual removal of denaturant, could lead to acceleration of the refolding rate of pepsin Although further studies are obviously necessary for the elucidation of factors determining these slow refolding processes, the achievement of *in vitro* refolding of pepsin in the present study might open up a new way to investigate refolding mechanisms of multidomain proteins.

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