RECOVERY OF ENZYMATIC ACTIVITY OF REDUCED TAKA-AMYLASE A AND REDUCED LYSOZYME BY AIR-OXIDATION

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Various proteins which consist of a single peptide chain often contain some disulfide bonds in the molecule. Such disulfide bonds are believed to be responsible for maintaining the protein molecule in the definite characteristic folded structure (1). It was suggested that, if these bonds were reductively cleaved and then the reduced protein was oxidized, the yield of regeneration of intact protein would be very low owing to the formation of many isomers and intermolecular aggregates (2). However, White found recently that the fully reduced and enzymatically inactive bovine pancreatic ribonuclease can be oxidized by atmospheric oxygen to produce a soluble protein in high yield with a specific activity approaching that of native RNase (3), and confirmed that the regenerated RNase possessed identical secondary and tertiary structure with those of native RNase (4). The present authors found that intact Taka-amylase A (Asp. oryzae α-amylase ; M.W. 52,000) can be regenerated from 8M urea-denatured Taka-amylase A, of which secondary and tertiary structure had been completely destroyed, by gradual removal of urea by dialysis against a buffer (5). We extended the study to the regeneration of intact Taka-amyl-

lase A from Taka-amylase A of which disulfide bonds were all reductively cleaved in 8M urea. Similar study was also carried out with hen's egg white lysozyme, accurate estimate of which activity became possible by a new method developed in our laboratory (6).

Taka-amylase A (0.5-1.0%) and lysozyme (0.2-1.0%) were reduced in the presence of excess sodium thioglycolate. 0.01M EDTA was added in the case of Taka-amylase A to sequester calcium ions which protect Taka-amylase A from denaturation. Molar ratio of thioglycolate to protein was about 900 and 400, respectively. Taka-amylase A was reduced at pH 6 and 20°C, and lysozyme at pH 9 and 30°C, both for 20 hours. After removing the remaining thioglycolate by passing through a column of Amberlite IRA-400 (acetate form) saturated with 8M urea, the amount of sulfhydryl groups formed by reduction was determined by spectrophotometric titration with PCMB. Some of the properties of the reduced enzymes are shown in Tab.1 with those of the native, reduced oxidized, and reduced-oxidized-crystallized enzymes.

Taka-amylase A and lysozyme contain four and five disulfide bonds per molecule, respectively and contain no sulfhydryl groups. It is clear that all disulfide bonds were reductively cleaved in both cases. After the removal of thioglycolate, the enzymatic activity was measured promptly by diluting the enzyme solution to such a low concentration of urea as the effect of urea can be neglected. The activity of Taka-amylase A was determined using amylose as substrate and that of lysozyme using glycol chitin as substrate (6). By comparing the properties of the reduced enzymes with those of native enzymes in Tab.1, it is evident that these reduced enzymes should be markedly unfolded.

Tab.1 Properties of Taka-amylase A and Lysozyme in Native, Reduced, Reduced Oxidized, and Reduced-Oxidized-Crystallized States

	SH (moles/mole enzyme)	$[\eta]^*$ (dl/g)	$[\alpha]^*_{500}$	enzymatic*** activity (%)
Taka-amylase A native	0.0	0.035****	20.5° (λ_c = 280mμ)	100
reduced	7.6	0.405**	147° (λ_c = 219mμ)	0
reduced oxidized	2.5	———	———	40
reduced oxidized crystallized	0.0	0.038****	19.4° (λ_c = 277mμ)	92
Lysozyme native	0.0	0.037****	75° (λ_c = 261mμ)	100
reduced	10.3	0.16**	121° (λ_c = 229mμ)	0
reduced oxidized	1.2	———	———	47

*$[\eta]$, $[\alpha]$ and λ_c were measured at 20° for Taka-amylase A and at 30° for lysozyme. **These values were measured in the presence of 8M urea + 0.1M thioglycolate. ***Activity is expressed as percent of the activity of the native enzymes in the equivalent concentration. ****in 0.1M KCl, pH 6.

Reoxidation was conducted as follows. Taka-amylase A was reoxidized merely by dialyzing the reduced Taka-amylase A in a Visking sac in an open vessel for two days against 2 liters of pH 8.0, 0.02M veronal buffer. The outer solution was stirred and exchanged twice in the course of experiment. The oxidation and removal of urea proceeded at the same time. No aggregate formation was observed even when Taka-amylase A concentration was as high as 1%. On the other hand, the reduced lysozyme

solution in 8M urea was diluted four times with water. After adjusting the pH of the solution to 8.0 by the addition of a small amount of 0.5M phosphate buffer, the solution was poured into a conical beaker to keep the surface of the solution sufficiently large and to make the layer thin. The solution was agitated by a magnetic stirrer to oxidize the reduced lysozyme by air. Aggregate formation was observed, and its amount increased with the lysozyme concentration. The yield of soluble protein was about 50% when lysozyme concentration was 0.05-0.1%.

As is clear from Tab.1, the amount of sulfhydryl groups decreased and the enzymatic activity was recovered in these reoxidized enzymes. To the reoxidized solution of Taka-amylase A calcium acetate was added to 0.01M in concentration. Then, an equal volume of cold acetone was added slowly to the solution to precipitate Taka-amylase A. The precipitate was dissolved in 0.02M calcium acetate. After removing insoluble residue by centrifugation, acetone was added slowly to the supernatant as in the crystallization of native Taka-amylase A and crystalline Taka-amylase A separated out. The yield was about 30% to native Taka-amylase A at the start of these treatments. Some properties of this crystalline Taka-amylase A were measured and coincided well with those of native Taka-amylase A as shown in Tab.1. Isolation of regenerated lysozyme is not yet attained.

When all the disulfide bonds of these enzymes are cleaved in 8M urea solution, the enzyme should assume the random coil of linear polypeptide. From these experiments metioned, it is clear that the active conformation of these enzymes (just like the native enzymes*) was regenerated by air-oxidation of these

*At least in the case of Taka-amylase A.

reduced enzymes after dilution or removal of urea. Because Taka-amylase A has molecular weight fairly higher than that of RNase and has been suggested to have compact and highly organized molecular structure (7-9), the findings, suggesting the regeneration of its secondary and tertiary structure, seems to be interesting. Further detailed studies are now in progress.

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