Cat. # 7350

For Research Use

TaKaRa

Refolding CA Kit

Product Manual

v202003Da

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I. Description

In microorganisms, such as *Escherichia coli*, a recombinant protein is not always produced in the soluble form protein with its activity, but instead is often accumulated as the insoluble inclusion body in the cells. In such cases, in general, the inclusion bodies are unfolded with denaturants such as urea or guanidine hydrochloride and then refolded by removing the denaturants by dialysis or dilution, to recover the activities of recombinant proteins. However, these operations for refolding are laborious and time-consuming, and moreover, the refolding efficiency is not very high. The method have been reported for improving the refolding efficiency by dividing the refolding operation into two steps to use the different compounds suitable for each step.

This kit is designed to examine the refolding conditions with combination of four surfactants and highly polymerized cycloamylose(CA), on employing this two-step operation, which provides the improved refolding efficiency, for the inclusion bodies of recombinant proteins.
This kit contains sufficient reagents to examine the conditions for 25 samples. Surfactants and CA are stable after 50 rounds of freezing and thawing. (However, the contamination of microbes or amylase into CA solution will reduce the refolding capacity because of the decomposition of CA.)
And this kit provides simple and rapid procedure, since all the operation can be performed in 1.5 ml microtubes.

II. Components (for 25 reactions)

1. 8 M Guanidine Hydrochloride (GdmCl)	1 ml x 2
2. 4 M Dithiothreitol (DTT)	50 μl
3. 1% Tween 40	1 ml x 2
4. 1% Tween 60	1 ml x 2
5. 1% CTAB (cetyltrimethylammoniumbromide)	1 ml x 2
6. 1% SB3-14 (myristylsulfobetaine)	1 ml x 2
7. 200 mM DL-Cystine	0.75 ml x 2
8. 3% CA (highly polymerized cycloamylose)	1.6 ml x 7

These reagents may precipitate during storage. In such cases, warm the solution at 37℃ and dissolve the precipitates completely. Since surfactants foam by vigorous agitation, spin down the solution after stirring with Vortex mixer.

III. Storage

-20℃
＊ 2 years from date of receipt under proper storage conditions.

IV. Principle

Addition of surfactant into the unfolded protein solution provides diluting the denaturant and protects the protein molecules from aggregation. Subsequently, the protein will be refolded in its authentic conformation and recover its activity by adding CA, which has inclusion ability to remove surfactant from the protein-surfactant complex.

V. Protocol

The following protocol is for unfolding the protein and for examining the refolding conditions by changing the surfactant to be used.

[Unfolding]

1. Suspend the inclusion bodies in an appropriate buffer. The protein concentration is recommended to be 10 mg/ml or less.

2. To 24 μl of the suspension of inclusion bodies, add 75 μl of 8 M Guanidine Hydrochloride and 1 μl of 4 M DTT, and allow it to stand at room temperature for 1 hour.

[Refolding]

3. To 20 μl of the unfolded protein solution, add 1.4 ml of a surfactant solution (+DL-Cystine)*, and allow it to stand at room temperature for 1 hour.
   * Surfactant solution is prepared by adding 1/20 volume (70 μl) of any of the surfactans contained in the kit (1% Tween 40, 1% Tween 60, 1% CTAB or 1% SB3-14) to an appropriate buffer (1.33 ml). If cystine is further added to the solution, 1/100 volume (14 μl) of 200 mM DL-cystine contained in the kit should be added.

4. To 400 μl of the reaction mixture in step 3, add 100 μl of 3% CA solution, and incubate at room temperature overnight.

5. Centrifuge at 15,000 rpm for 10 minutes, and collect the supernatant.

VI. Appendix

1 Denaturation and Refolding of Lysozyme

The lysozyme (15 mg/ml in water; 24 μl) was unfolded by adding the denatuant, as described in protocol V. To 20 μl of this unfolded protein solution, 1.4 ml of cationic surfactant solution (Tris-acetate buffer, pH 7.6, containing 0.05% CTAB and 2 mM cystine) was added, followed by incubation at room temperature for 1 hour. Subsequently, CA was added at different concentrations to the reaction mixture, and refolding was performed by incubation at room temperature for 16 hours (overnight). After centrifugation, the supernatant was collected to determine the enzyme activity.

[Determination of the lysozyme activity]

Reaction mixture for assay :

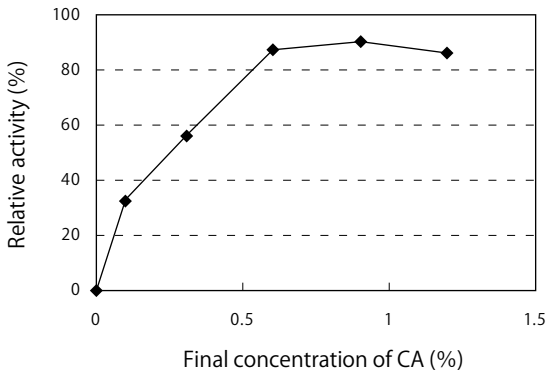
Micrococcus lysodeikticus dried cells (0.16 mg/ml) in 50 mM sodium phosphate buffer (pH 6.2).

Refolded lysozyme was added into this reaction mixture and the changes in absorbance at 450 nm were measured over time. The relative activity was determined, based on the activity of the lysozyme diluted in the buffer at the same ratio without any denaturation treatment taken as 100%.

[Results]

Final concentration of CA (%)	Relative activity (%)
0	0
0.1	33.3
0.3	56.7
0.6	88.3
0.9	90.8
1.2	87.1

The final concentration of CA recommended in this protocol is 0.6%.



The relative activity of lysozyme was 88%, when CTAB was used as a surfactant and CA was used at the final concentration of 0.6% (recommended in this protocol).

2. Denaturation and Refolding of Citrate Synthase

Citrate synthase (2.4 mg/ml in ammonium sulfate; 24 μl) was unfolded by adding the denaturant, as described in protocol V. To 20 μl of this unfolded protein solution, 1.4 ml of non-ionic surfactant solution (Tween 40 or Tween 60 at the final concentration of 0.05% in 158 mM Tris-HCl, pH 7.6) was added, followed by incubation at room temperature for 1 hour. Subsequently, CA was added at different concentrations to the reaction mixture, and refolding was performed by incubation at room temperature for 16 hours (overnight). After centrifugation, the supernatant was collected to determine the enzyme activity.

[Determination of the activity]

Reaction mixture for assay :

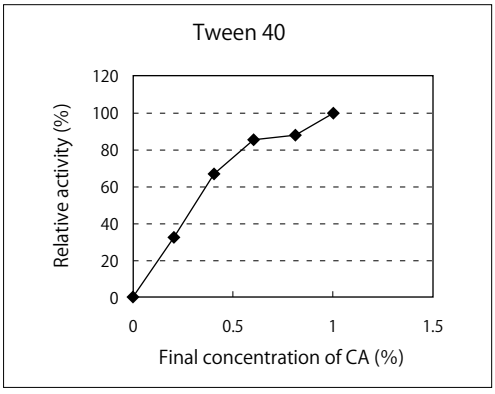
158 mM	Tris-HCl (pH 7.6)
0.023 mM	acetyl-CoA
0.5 mM	oxaloacetic acid
0.12 mM	5, 5'-dithiobis (2-nitrobenzoic acid)

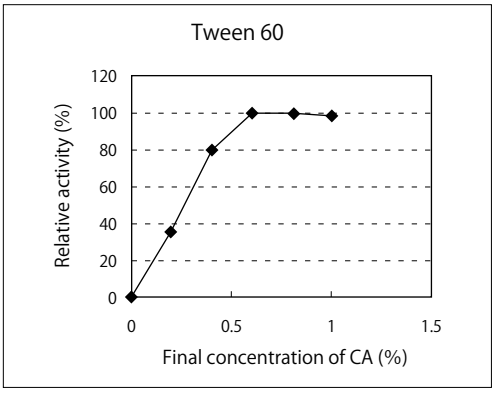
Refolded citrate synthase was added to the reaction mixture and the changes in absorbance at 412 nm were measured over time. The relative activity was determined, based on the activity of the citrate synthase diluted in the buffer at the same ratio without any denaturation treatment taken as 100%.

[Results]

Final concentration of CA (%)	Relative activity (%)	
	Tween 40	Tween 60
0	0	0
0.2	33.1	34.7
0.4	66.2	80.0
0.6	86.2	100
0.8	88.5	100
1.0	100	98.0

The final concentration of CA recommended in this protocol is 0.6%.





The relative activities of citrate synthase were 86% and 100%, respectively, when Tween 40 or Tween 60 was used as a surfactant and CA was used at the final concentration of 0.6% (recommended in this protocol).

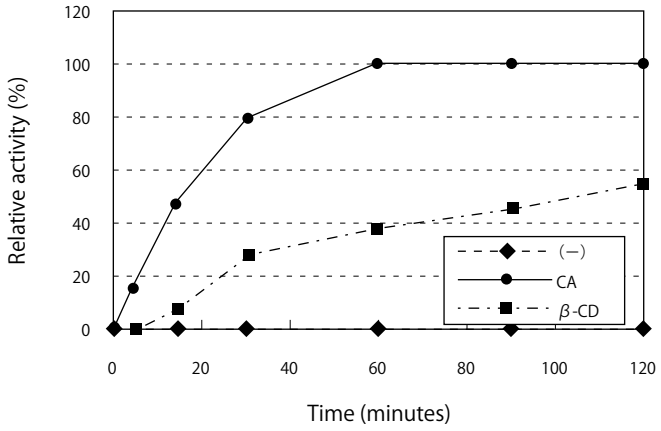
3. Investigation of the Time Period Required for Refolding

To unfolded Citrate Synthase, Tween 60 buffer was added, followed by incubation at room temperature for 1 hour. Sebsequently, CA or β-cyclodextrin (β-CD)* was added. The enzyme activity was determined over time to study the time required for refolding.

* cyclic α-1, 4-glucan with 7-degree of polymerization

[Result]

Time (minutes)	(－)	CA (Final concentration 0.6%)	β-CD (Final concentration 0.6%)
0	0	0	0
1	0	0	0
5	0	15.6	0
15	0	47.8	7.8
30	0	78.6	28.3
60	0	100	38.6
90	0	100	46.2
120	0	100	54.8



Time-course of refolding

The result shows that 100% of the enzyme protein was refolded by one hour incubation using CA, while about 40% was refolded by similar incubation using β-CD. Although overnight incubation is recommended for refolding in the protocol of this kit, it is possible that the incubation time is shortened for refolding using CA under certain conditions.

VII. Q & A

Q1 ; How to scale up for refolding and how refolding ratio is?
A1 ; As this kit is for examining the refolding conditions, it is not suitable for treatment at large scale of protein. When 10 mg/ml protein solution added guanidine hydrochloride do not become viscous, unfolding process is done well. The refolding will occur at 100% usually.

Q2 ; Why were white precipitates appeared again in the refolded protein solution collected by centrifugation.
A2 ; During refolding, CA forms the clathrate compounds (white flocculation) with the surfactant. This clathrate compounds by can be removed centrifugation, . This operation does not affect the structure of the refolded protein.

Q3 ; How are the refolded proteins detected?
A3 ; In many cases, the refolded protein solution collected as supernatant can be used directly for detection of the activity.
The recombinant proteins fused with Tag sequences such as His-Tag or GST-Tag can be purified by affinity chromatography utilizing the Tag.

Q4 ; How to large scale refolding?
A4 ; Refolding CA Large Kit (Cat. #7351) is recommended.

VIII. References

1) Daugherty D L, Rozema D, Hansen P E, and Gellman S H. *J Biol Chem*. (1998) **273**: 33961-33971.
2) Sundari C S, Raman B, and Balasubramanian D. *FEBS lett*. (1999) **443**: 215-219.
3) Machida S, Ogawa S, Xiaohua S, Takaha T, Fujii K, and Hayashi K. *FEBS lett.* (2000) **486**: 131-135.

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