Introduction of developed products

r-Trypsin immobilized resin for insulin production

Abstract

This is an enzyme immobilized resin which recombinant trypsin used for insulin production is immobilized on cellulose particles. Since trypsin is covalently bound to cellulose particles, trypsin activity is much more stable than free trypsin. From this feature, it can be expected to reduce the manufacturing cost by repeated use of trypsin digestion.

Feature of r-Trypsin immobilized resin

Ligand	Recombinant Trypsin (r-porcine trypsin from E.coli)
Base resin	Cellulose particles
Particle size	Ave. 90 μ m
Enzyme activity	＞ 2,000 BAPNA (Benzyl-DL-arginine-p-nitoroanilide) U/ml
Preservation solution	PBS – 50 % Glycerol

How to handle developed products

The r-trypsin-immobilized resin is an under developing product. The quality of the Cellufine which are chromatography media are guaranteed by ISO 9001, but the quality of this product is subject to change as it is under development.

This product is not sold. We provide a small amount of samples as a developing product. For pharmaceutical companies, diagnostics companies or other manufacturing companies and researchers who would like to handle this product for manufacturing process, we will provide samples those who consenting to above fact.

For inquiries regarding samples, please contact the following.

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http://www.jnc-corp.co.jp/fine/en/cellufine/

■Excellent enzyme activity with various pH conditions

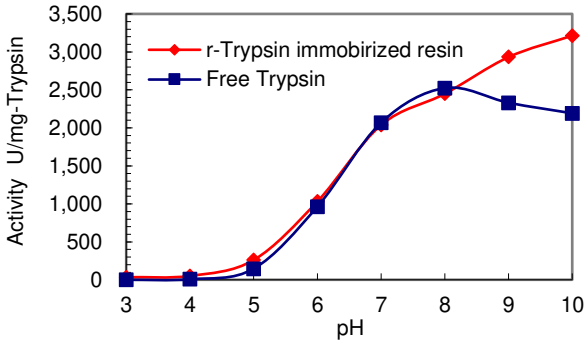
Measurement conditions

Reaction buffer: Buffer of pH 3-10 + 10 mM CaCl2

Reaction temperature: 25 °C

Enzyme substrate: BAPNA (Benzoyl-DL-arginine-p-nitranilide)

The enzyme activity of the r-trypsin immobilized resin was evaluated for each pH condition. By immobilizing trypsin on the resin, trypsin activity was maintained even under highly alkaline conditions such as pH 10. On the other hand, the free trypsin became optimally active at pH 8, and lost activity under high pH conditions.



■ Excellent durability in high temperature conditions

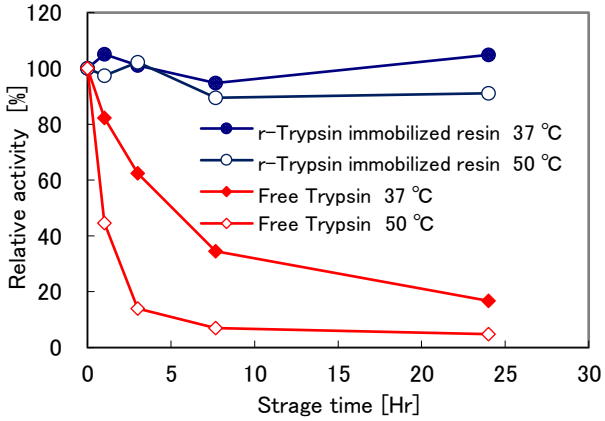
Measurement conditions

Reaction buffer: 50 mM Tris-HCl 10 mM $CaCl_2$ (pH 8.0)

Temperature: 37 °C or 50 °C

Enzyme substrate: BAPNA

The r-trypsin immobilized resin can be used under high temperature conditions compared to the free Trypsin. It maintained trypsin activity even under high temperature conditions of 50 °C. On the other hand, the free Trypsin lost activity at 37 °C due to its Autolysis. It can be seen that the r-trypsin immobilized resin suppresses autolysis by immobilizing trypsin on the resin.



■Excellent long-term storage stability

Storage conditions

・Preservation solution: PBS + 50% Glycerol

・Storage temperature : 4 °C

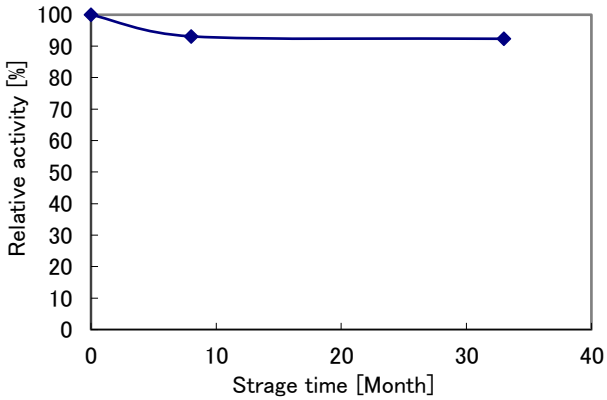
Measurement conditions

Reaction buffer: 50 mM Tris-HCl 10 mM CaCl2 (pH 8.0)

Reaction temperature: 25 °C

Enzyme substrate: BAPNA

The r-trypsin immobilized resin showd excellent storage stability. It was stored in 4 °C and maintained trypsin activity for 33 months.



■Trypsin activity in organic solvents

Measurement conditions

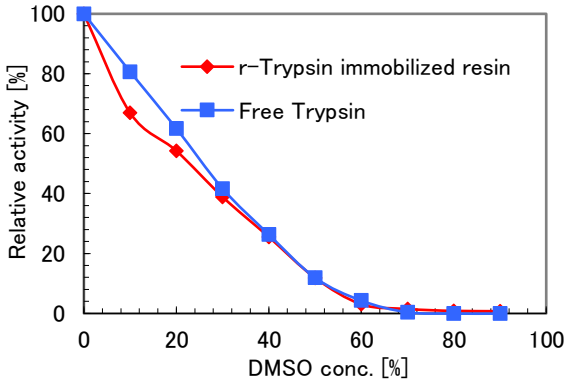
Reaction buffer: 50 mM Tris-HCl,10 mM $CaCl_2$, pH8.0
+ 0 – 90 % of organic solvent

Reaction temperature: 25 °C

Enzyme substrate: BAPNA

Organic solvents used in insulin production process include dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF) and acetonitrile. Even the buffer containing these organic solvents, r-Trypsin immobilized resin showed the same trypsin activity as the free Trypsin. From this result, it found that the r-trypsin-immobilized resin can be preferably used as an alternative for the free Trypsin for insulin production.

<DMSO>



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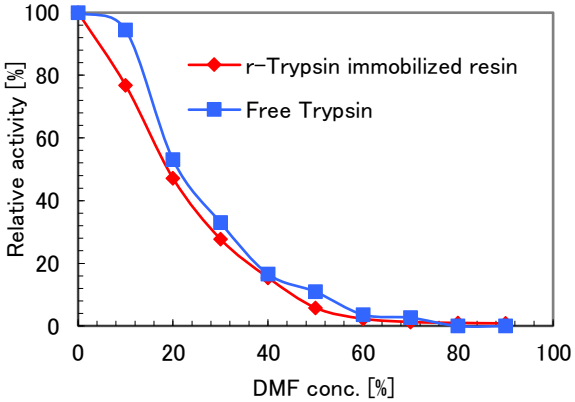

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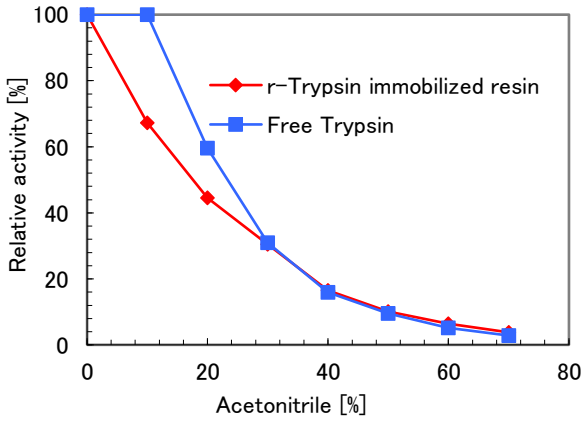


■ Clean-in-place (CIP) of r-trypsin immobilized resin

The r-Trypsin immobilized resin was packed to the column and clean-in-place resistance of the resin was evaluated .

Measurement conditions

Column: 0.5 cm I.D. x 5 cm (1 ml)

Enzyme substrate: BAPNA

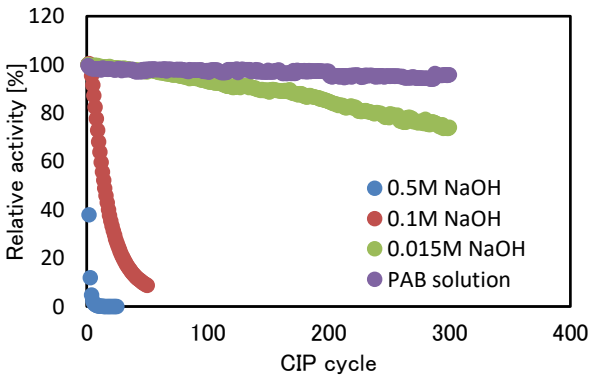
Reaction temperature: 25 °C

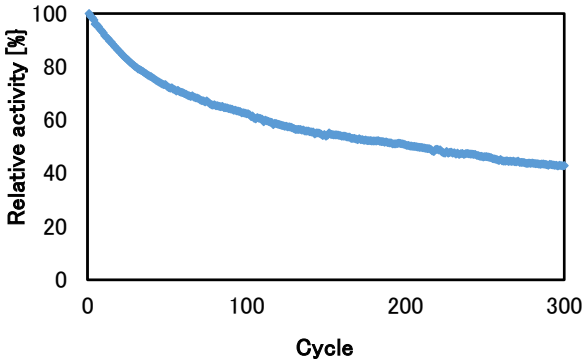
Activity evaluation: Relative activity = (Cleavage substrate peak area in cycle N) / (Cleavage substrate peak area in the first cycle)×100

Column program

Equilibration	5 CV	50mM Tris-HCl, 10mM $CaCl_2$, pH8.0
Sample load	10 μl	10mM BAPNA in DMSO
Elution	5 CV	Equilibration buffer Detection: PDA detector 410 nm
CIP	5 CV	NaOH (0.015 M, 0.1 M, 0.5 M)または PAB solution： 132mM phosphoric acid , 184mMacetic acid, 2.4%(v/v)benzyl alcohol, pH1.6
Wash	5 CV	Equilibration buffer

CV: Column volume





Sodium hydroxide (NaOH) is generally used for CIP of chromatography columns. The r-Trypsin immobilized resin has the general characteristics found in protein immobilized resin, that it has low resistance to NaOH because it immobilizes the protein. However, when CIP with 0.015 M NaOH, it maintained 85% activity in 200 cycles test. In a test using an acidic CIP solution PAB, the activity was maintained even after 300 clean-in-place cycles.

■Durable test with lysine buffer, pH 12

Measurement conditions

Column: 0.5 cm I.D. x 5 cm (1 ml)

Enzyme substrate: BAPNA

Reaction temperature: 25 °C

Activity evaluation: Relative activity = (Cleavage substrate peak area in cycle N) / (Cleavage substrate peak area in the first cycle)×100

Column program

Equilibration	5 CV	0.1 M Lysine buffer (pH12)
Sample load	10 μl	10mM BAPNA in DMSO
Elution	5 CV	Equilibration buffer Detection: PDA detector 410 nm
CIP	5 CV	PAB solution
Wash	5 CV	Equilibration buffer

A pH 12 lysine buffer may be used for trypsin treatment. Since the reaction is highly alkaline, the enzyme activity is reduced. In this study, 80% activity was maintained after 30 cycles repeated uses. After 300 cycles repeated uses, the activity decreased to 43%.

■Durable test with 50 % DMF

Measurement conditions

Column: 0.5 cm I.D. x 5 cm (1 ml)

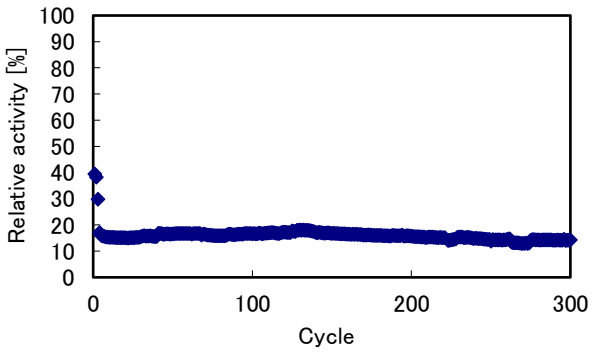
Enzyme substrate: BAPNA

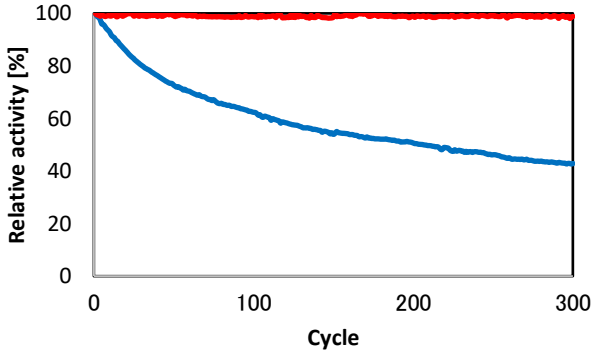
Reaction temperature: 25 °C

Activity evaluation: Relative activity = (Cleavage substrate peak area in cycle N) / (Cleavage substrate peak area in the first cycle)×100

Column program

Equilibration	5 CV	50% DMF in 0.5M Tris-HCl, pH8.7
Sample load	10 μl	10mM BAPNA in DMSO
Elution	5 CV	Equilibration buffer Detection: PDA detector 410 nm
CIP	5 CV	PAB solution
Wash	5 CV	Equilibration buffer





Basically, in the condition of 50% DMF, trypsin activity is only 10-20%. The activity in the repeated use test after the column packing also behaved in the same way.

After 300 cycles repeated tests, the trypsin activity was evaluated with a buffer (50 mM Tris-HCl, 10 mM CaCl2, pH 8.0) excluding DMF, then the enzyme activity recovered to 100%. This result indicates that trypsin activity is maintained even after repeated use up to 300 cycles using 50% DMF.

■Durable test with high alkaline conditions

Measurement conditions

Column: 0.5 cm I.D. x 5 cm (1 ml)

Enzyme substrate: BAPNA

Reaction temperature: 25 °C

Activity evaluation: Relative activity = (Cleavage substrate peak area in cycle N) / (Cleavage substrate peak area in the first cycle)×100

Column program

Equilibration	5 CV	0.1M Lysine buffer, pH9 or pH10
Sample load	10 μl	10mM BAPNA in DMSO
Elution	7.5CV	Equilibration buffer Detection: PDA detector 410 nm
CIP	5 CV	PAB solution
Wash	5 CV	Equilibration buffer

The pH 9 lysine buffer maintains trypsin activity after 300 cycles test. On the other hand, pH 12 lysine buffer caused an irreversible change in enzyme activity and decreased activity.

■Durable test with acetonitrile

Ⅰ) 50 % acetonitrile / Tris buffer, pH 8

Measurement conditions

Column: 0.5 cm I.D. x 5 cm (1 ml)

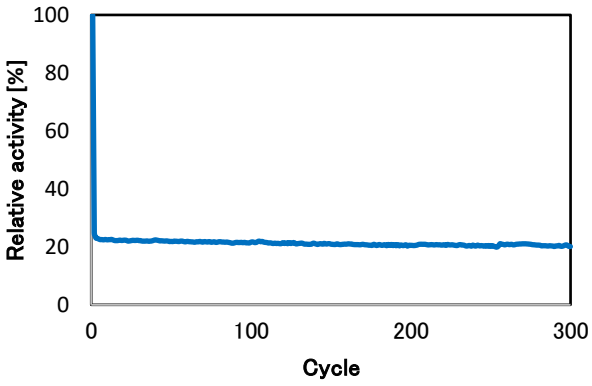
Enzyme substrate: BAPNA

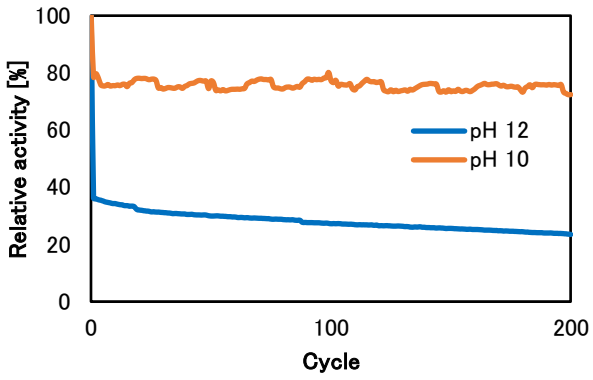
Reaction temperature: 25 °C

Activity evaluation: Relative activity = (Cleavage substrate peak area in cycle N) / (Cleavage substrate peak area in the first cycle)×100

Column program

Equilibration	5 CV	50 % acetonitrile / 50 mM Tris-HCl, 10 mM CaCl2 (pH 8) (V/V)
Sample load	10 μl	10mM BAPNA in DMSO
Elution	7.5CV	Equilibration buffer Detection: PDA detector 410 nm
CIP	5 CV	PAB solution
Wash	5 CV	Equilibration buffer





Trypsin activity in the presence of 50% acetonitrile is only 20%. The activity in the repeated cycle test after the column packing also behaved in the same way.

After 300 repeated tests, the enzyme activity was evaluated with a buffer (50 mM Tris-HCl, 10 mM CaCl2, pH 8.0) excluding acetonitrile, then the enzyme activity recovered to 100%. This result indicates that trypsin activity is maintained even after repeated use up to 300 times using 50% acetonitrile.

Ⅱ) 20 % Acetonitrile / Lysine buffer, pH 10 or 12

Measurement conditions

Column: 0.5 cm I.D. x 5 cm (1 ml)

Enzyme substrate: BAPNA

Reaction temperature: 25 °C

Activity evaluation: Relative activity = (Cleavage substrate peak area in cycle N) / (Cleavage substrate peak area in the first cycle)×100

Column program

Equilibration	5 CV	20 % acetonitrile / 50 mM lysine buffer, 10 mM CaCl2, pH 10 or 12 (V/V)
Sample load	10 μl	10mM BAPNA in DMSO
Elution	7.5CV	Equilibration buffer Detection: PDA detector 410 nm
CIP	5 CV	PAB solution
Wash	5 CV	Equilibration buffer

Under the condition of 20% acetonitrile (pH 10), trypsin activity was maintained at less than 80%. After 200 cycles test, the enzyme activity was evaluated with a buffer (50 mM Tris-HCl, 10 mM CaCl2, pH 8.0) excluding acetonitrile, then the enzyme specific activity recovered to 100%.

On the other hand, in repeated tests using 20% acetonitrile, pH 12, which is a highly alkaline condition, trypsin activity decreased depending on the number of cycles of use. After 200 cycles test, trypsin activity was evaluated with a buffer (50 mM Tris-HCl, 10 mM CaCl2, pH 8.0) excluding acetonitrile. As a result, the enzyme activity was not recovered, and the enzyme activity was lost due to irreversible changes.

■Stability with organic solvents

Measurement conditions

Storage buffer:

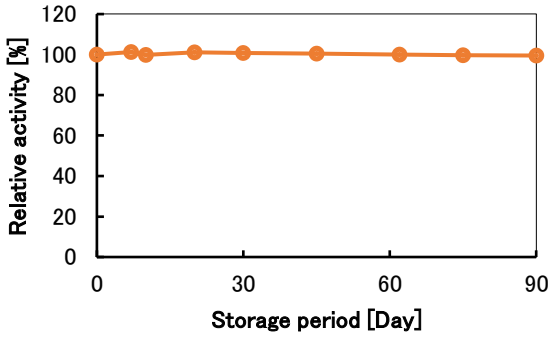
- 50 % DMF / 50 mM Tris-HCl – 10 mM $CaCl_2$ (pH 8)
- 50 %DMSO / 50 mM Tris-HCl – 10 mM $CaCl_2$ (pH 8)
- PAB solution

Storage period： 90 day, 25 °C

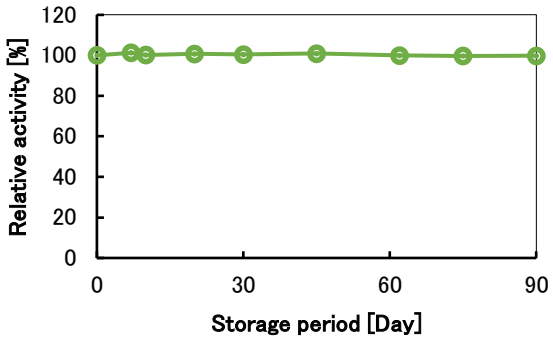
Enzyme activity evaluation:

Packing the resin to 1 ml column. Evaluation using BAPNA as a substrate under the conditions of 50 mM Tris-HCl, 10 mM CaCl2, pH 8.

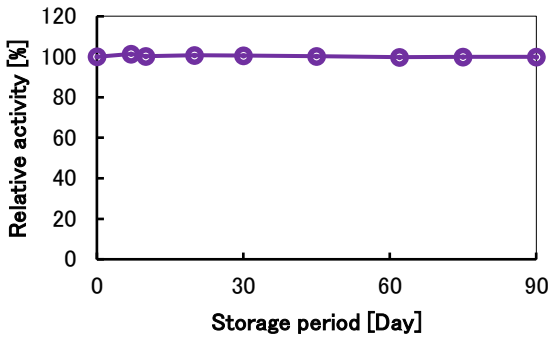
A) 50% DMF



B) 50% DMSO



C) PAB solution



After storing the r-Trypsin immobilized resin in each storage solution for a certain period, the trypsin activity was evaluated. The activity was maintained in each case. It was found that the r-Trypsin immobilized resin is an enzyme immobilized resin that is highly resistant to organic solvents.

Appendix

Method for measuring trypsin activity

Reagents:

Benzoyl-DL-arginine p-nitroanilide (BAPNA)

Reaction buffer: 50 mM Tris-HCl, 10mM $CaCl_2$ (pH8.0)

The above buffer is a basic condition of this document.

BAPNA soulution;

Dissolve 100 mM BAPNA in dimethyl sulfoxide

Reaction stop buffer;

33% acetic acid

Measurement method:

1) Prepare a wet resin by removing the preservation solution with a filter.
2) Add 0.2 g of wet resin and 980 ul of reaction buffer to a 2 mL microtube and stir gently at 25 °C for 3 minutes.
3) Add 10 µL of BAPNA solution to the microtube.
4) Settle at 25 ° C for 10 minutes. Then add 200 uL of reaction stop buffer to stop the reaction.

Regeneration method:

In the column or with filter, wash with 5-10 column volumes of reaction buffer.

Storage

Equilibrate the resin with PBS (pH 7) – 50% glycerol.

Store at 4 ° C.