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Porcine-Enterokinase

Store at -20°C

Cat. No.	Description	Quantity
G699	Porcine-Enterokinase, Light Chain	100 units in 100µl

Product Description and Application

The Porcine-Enterokinase included in this kit is the catalytic subunit of the native holoenzyme, and is highly active and specific for cleaving fusion proteins with the recognition sequence, DDDDK, in the interdomain linker. The Porcine-Enterokinase is a recombinant glycoprotein derived from porcine source sequence, and is produced from mammalian expression system. The purified Porcine-Enterokinase behaves as a 47kD band under denaturing and reducing conditions as visualized on SDS-PAGE.

Porcine-Enterokinase is a site-specific protease that recognizes and cleaves after the C-terminal end of lysine residue in the recognition sequence, DDDDK. Unlike other site-specific proteases that cut within the recognition sequences leaving extra amino acids in the cleaved peptide products, the C-terminal peptide fragment produced from the Porcine-Enterokinase cleavage reaction doesn't inherent any residues from the DDDDK recognition sequence*. Therefore, the application can be extremely advantageous for producing a 100% native protein sequence and structure from recombinant fusion protein, which has the desired product immediately after the enterokinase recognition sequence, DDDDK.

*Note: Porcine-Enterokinase will not cleave at sites where lysine is followed by proline.

In addition, DDDDK is part of the octapeptide FLAG tag (DYKDDDDK), which has been used as a fusion tag for recognition by antibody and detection of fusion protein expression with Western blot analysis, and for purification of the fusion protein by Anti-FLAG affinity chromatography. This array of applications makes enterokinase an ideal tool in the research involving the study of protein structure and function, and protein production where native protein structures and sequences are desired.

Kit Components

Component	Volume	Storage Buffer
Porcine-Enterokinase	100 units in 100µl	2mM $CaCl_2$ 20mM Tris-HCl (pH8.0) 200mM NaCl 50% Glycerol(v/v)

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Recommended Reaction Buffer

Component	Buffer Recipe
10X Reaction Buffer	20mM $CaCl_2$ 500mM NaCl 200mM Tris-HCl (pH8.0)

Shipping and Storage

Upon arrival, Porcine-Enterokinase should be stored at -20°C. Avoid repeated freeze-thaw cycles to retain maximum performance. All kit components are stable for 1 year from the date of shipping if stored and handled properly.

Cleavage Reaction

To determine the optimal cleavage condition of the target protein by Porcine-Enterokinase, a sufficient amount of the target protein (i.e., 150µg with at least 1mg/ml of concentration) is needed at the beginning. Provided that a sufficient amount of protein substrate is present in the reaction (i.e., 0.4mg/ml), the parameters that influence the cleavage efficiency includes 1) final concentration of Porcine-Enterokinase in the reaction, 2) reaction temperature, 3) reaction time, and 4) composition of buffer in the reaction. These parameters vary with different target proteins because each target protein presents the DDDDK cleavage sequence differently in their three-dimensional structures in solution. The following recipe is an example of a grid search, composed of a number of small-scale digestion reactions. It is designed to estimate the optimal enterokinase concentration in the reaction, and at the same time find out the length of time needed for the reaction to finish.

Protocol

1. Make dilutions of Porcine-Enterokinase in Storage Buffer (recipe provided in 'Kit Components' table) to produce solutions having 0.01, 0.04, 0.1, 0.4, and 1 U of enterokinase per µl.

2. Combine the following components in the labeled tubes.

Component	Volume
10X Reaction Buffer	5µl
Target Protein	20µg
Diluted Porcine-Enterokinase	5µl
ddH_2O	to 50 µl
Total Volume	**50µl**

[Note: Follow the recipe below to make a Negative Control (no enterokinase present) in which 5µl of Storage Buffer is used in place of enterokinase.]

3. Incubate the reaction at room temperature (e.g., 25°C).

4. Take 10µl aliquots into 10µl 2X SDS-PAGE sample/loading buffer after 2, 9, and 24 hours.

5. Determine the extent of cleavage by SDS-PAGE analysis. [Note: Two SDS-PAGE gels are needed as there will be 15 lanes for the cleavage reactions, plus additional lanes for the negative controls and molecular weight markers.]

Our EK Cleavage Control Protein

Our cleavage control protein, to which the DDDDK enterokinase recognition sequence is engineered to a flexible interdomain linker, is a purified recombinant glycoprotein with an estimated molecular weight of 53kD on SDS-PAGE under denaturing and reducing condition. Digestion of the cleavage control protein by Porcine-Enterokinase results in an overlapping single band of approximately 30kD on SDS-PAGE (Figure 3).

Technical Notes

1. It is best to maintain the target protein concentration at high level in the reaction. For Porcine-Enterokinase the rate of target protein cleavage is directly dependent on and linearly proportional to the substrate concentration in the reaction. Keeping the substrate concentration at high level ensures that enterokinase is working at its best reaction velocity possible. For this consideration, at least 0.5mg/ml of target protein concentration in the reaction is needed for the parameter optimization procedure mentioned above. When a satisfactory result with optimized reaction parameters is determined, scales up the reaction components proportionally (i.e., maintain the same concentration of buffer, target protein substrate, and Porcine-Enterokinase in the scaled-up reaction volume).

2. Denaturants or chaotropic agents (such as the commonly used urea or guanidine hydrochloride) may increase the accessibility of the DDDDK cleavage site that could otherwise be sterically hindered, and facilitate the cleavage of target proteins. However, when denaturants is present at high level in the reaction (e.g., >2M of urea), enterokinase will be inhibited (Baratti et al., 1973). Target protein that contains denaturants can still be tested for enterokinase cleavage using the small-scale optimization procedure to confirm reaction efficiency and cleavage specificity.

3. Strong detergents such as SDS are denaturants. Depending on the target protein, significant level of unspecific cleavage at secondary sites was observed when SDS is present at 0.01% (w/v) in the reaction. Triton X-100 is a mild detergent, and when used as high as 1% (w/v) in the reaction Triton X-100 doesn't appear to affect enterokinase reaction efficiency or cleavage specificity.

4. The Porcine-Enterokinase is active at 4, 25, and 37°C tested. During the optimization of temperature parameter, one should consider the target protein stability at high temperature as the priority, and choose low temperature to ensure the maintenance of protein structure and function during and after cleavage.

5. Excess amount of imidazole (>50mM) and/or NaCl (>250mM) reversibly interferes and inhibits enterokinase activity. Presence of >2M NaCl in the reaction inhibits enterokinase completely. Porcine-Enterokinase, which has 5 disulfide bridges in the structure (Lu et al., 1999), is inhibited by the presence of reducing agents (e.g, β-mercaptoethanol).

In addition, enterokinase is inhibited by acidic or alkaline pH conditions (e.g., pH values below 6 or above 9). These commonly used buffer chemicals (e.g., imidazole, NaCl, reducing agents, and extremes of pH values) in the purification of target fusion protein substrate can be removed through buffer exchange, such as dialysis, ultrafiltration, or size-exclusion chromatography.

6. To prevent further degradation of the target protein after the reaction is finished, removal of enterokinase is recommended. Enterokinase can be removed by filtering the reaction mixture through affinity chromatography - Soybean Trypsin Inhibitor Agarose or Benzamidine Sepharose resins. In addition, inactivation of enterokinase can be done with PMSF or APMSF, which irreversibly modifies the catalytic residues in the active site in enterokinase.

Troubleshooting

1. Extra product bands on SDS-PAGE - Unspecific cleavage of the substrate. Enterokinase is a sequence specific protease; however, it may exhibit low level of unspecific cleavage when reaction condition is not optimized (Maroux et al., 1971; Choi et al., 2001). In addition, excess level of enterokinase can also lead to unspecific digestion of the protein substrate. In order to minimize unspecific cleavage, optimize the reaction condition as outlined in this reaction protocol for each target fusion protein, and use minimal amount of enterokinase in the scale-up reaction.

2. Poor cleavage efficiency or no cleavage at all. First of all, when the DDDDK recognition site is immediately followed by proline, no cleavage is possible. Secondly, presence of protease inhibitors (e.g., benzamidine and/or PMSF) also inactivates enterokinase. Enterokinase is a serine protease in the chymotrypsin protease family, and is irreversibly inactivated by PMSF. Exchange the storage buffer condition for the target protein to remove any protease inhibitors present, and repeat the cleavage reaction. Thirdly, depending on the accessibility of the recognition sequence to enterokinase, the cleavage site may be obstructed/hidden inside the three-dimensional structure of the target protein, and therefore not accessible to the enterokinase's active site. Increasing the level of denaturant in the reaction as well as increasing the level of enterokinase can enhance the cleavage efficiency and may circumvent the difficulties in digestion. However, it is recommended that each reaction parameter be individually optimized in small-scale test reactions for the target fusion proteins in question.

References

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