Enterokinase cleavage is carried out at an enzyme/substrate ratio (w/w) of 1/20-1/200 for 1-24 h. For each fusion protein pilot experiments should be done to find out suitable conditions. The optimal pH for the reaction is between 7.0-8.0, but the enzyme can be used within pH 6.0-8.5. Suitable buffers are, 50 mM Tris* or 50 mM Mes*. In phosphate buffer the activity is significantly reduced. Higher concentrations of salts, e.g. NaCl, inhibit the reaction. Addition of CaCl$_2$ to the incubation mixture does not increase the reaction rate (4). Unsoluble aggregates of fusion proteins are cleaved extremely slowly or not at all. Denaturing of the fusion protein might be necessary to increase the accessibility of the cleavage site. Suitable denaturing agents are listed in the table. On application of urea it is recommended to also add 20 mM methylamine. In order to achieve a suitable concentration of the denaturing agent in the reaction mixture, the protein has to be correspondingly diluted with incubation buffer.

1. Adjust the concentration of the fusion protein to 0.3-1 mg/ml and a pH between 7.0-8.0. If the fusion protein is dissolved in phosphate buffer or in case of high ionic strength, it is necessary to exchange the incubation buffer either by dialysis or gel filtration.

2. Dissolve the content of a vial of enterokinase in redist. water to obtain a concentration of 0.25-0.3 mg/ml. If necessary, a further dilution should be done using the incubation buffer.

3. Pilot experiments: Carry out the pilot experiments with a small portion of your fusion protein.

   Incubate 25 μg fusion protein (25 μl with 1 mg/ml) with 0.6 μg enterokinase (2 μg with 0.3 mg/ml). Take 5 μg samples of the reaction mixture after 1, 3, 6 and 24 h, add 5 μl 2 x concentrated SDS-PAGE sample buffer, boil for 5 min and store at −15 to −25°C until SDS-PAGE is performed. To detect a possible unspecific cleavage either by autolysis or by proteolytic contaminations of the fusion protein a control incubation without enterokinase is recommended. To optimize the amount of enterokinase necessary for 100% cleavage do a similar experiment with variations of the enzyme concentration and 3 h incubation time.

   Analyse the samples of an experiments, the corresponding control incubations and a sample of uncut fusion protein on a SDS-PAGE gel (5).
4. Incubate the main portion of the fusion protein using the optimized conditions deduced from the pilot experiments. Check for complete cleavage by SDS-PAGE and use as reference a sample of uncut fusion protein.

![Image of SDS-PAGE](image)

**Fig.**: Cleavage of a fusion protein with enterokinase. Incubation was carried out in 50 mM Tris buffer, pH 8.0, as described for the pilot experiments in the procedure. Lanes 1, 8: calibration proteins, combithek*; lane 2: uncut fusion protein; lanes 3, 4, 5, 6, 7: incubations of the fusion protein with enterokinase for 1, 3, 6, 20 and 32 h respectively.

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>Concentrations tested</th>
<th>Inhibition of cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (+ methylamine)</td>
<td>0.1 - 3 M</td>
<td>none</td>
</tr>
<tr>
<td>SDS*</td>
<td>0.01 - 1%</td>
<td>inhibitory at 0.01%</td>
</tr>
<tr>
<td>Hydrogenated Triton X-100</td>
<td>0.01 - 1%</td>
<td>none</td>
</tr>
<tr>
<td>Tween 80*</td>
<td>0.01 - 1%</td>
<td>none</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>5%, 10%</td>
<td>none</td>
</tr>
</tbody>
</table>

**Tab.**: Influence of different denaturing agents on the cleavage rate of enterokinase. Incubation was carried out according to the procedure in 50 mM Tris buffer, pH 8.0, for 1 h at 37°C; amount of enterokinase: 1/40 (w/w) of the fusion protein.

**References**


* available from Roche Applied Science
Triton X-100 is a Trademark of Rohm & Haas, Philadelphia/USA.
Tween 80 is a Trademark of ICI Americas Inc., Wilmington/USA.

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