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(54) Title: HIGHLY PURIFIED PROTEINASE K

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A solid proteinase K preparation insoluble in water and obtainable by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, and isolating the precipitated proteinase K has a high purity.

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(54) Title: HIGHLY PURIFIED PROTEINASE K

(57) Abstract: A solid proteinase K preparation insoluble in water and obtainable by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, and isolating the precipitated proteinase K has a high purity.

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Highly purified proteinase K

Field of the invention

The present invention relates to a highly purified solid proteinase K preparation, a water-insoluble form of proteinase K; methods for its production and use of a water-insoluble form of proteinase K.

Background of the invention

Proteinase K (EC3.4.21.64) is a serine protease which can be isolated from the fungus Tritirachium album limber (Ebeling, W., et al., Eur. J. Biochem. 47 (1974) 91-97; and German Patent DE 19 65 281). Proteinase K is capable of digesting native keratin and remains active in the presence of low concentrations of SDS. Therefore, proteinase K is very useful for the isolation of native RNAs in high yields (Wiegers, U., and Hilz, H., Biochem. Biophys. Res. Commun. 44 (1971) 513-519).

Proteinase K is also widely used in the field of PCR. In such methods there are used, in intermediate steps, certain enzymes such as restriction endonucleases, polymerases and DNA modifying enzymes. It is often necessary to inactivate these enzymes before performing the next PCR step. Such inactivation is preferably performed by the use of proteinase K. It is therefore necessary that proteinase K is of high purity and is especially not contaminated with desoxyribonuclease, endonucleases, exonucleases and DNA.

Summary of the invention

It was surprisingly found that time-controlled precipitation of proteinase K leads to a water-insoluble product characterized by a solubility of 0% in buffer A (10 mM TrisHCl pH 7.5) or buffer B (5 mM Ca-acetate pH 7.5) and by a solubility of 100% in buffer A containing 50% glycerol.

The invention therefore comprises a solid proteinase K preparation which is insoluble in water and obtainable by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, and isolating the precipitated proteinase K.

The invention also comprises a method for the preparation of proteinase K in a solid form by ammonium sulfate precipitation, characterized in that an aqueous solution of proteinase K is treated with a saturated aqueous solution of ammonium sulfate or with solid ammonium sulfate crystals under such conditions that proteinase K precipitates, characterized in that ammonium sulfate is added to said aqueous solution of proteinase K in that the ammonium sulfate concentration in said solution increases in an amount of 0.1 to 0.2 M per 5 minutes up to a final concentration of 1.5 M to 2 M after 1 hour and the formed proteinase K precipitate is isolated.

In a preferred embodiment of the invention, washing of the precipitate is performed with water or an aqueous buffer solution (pH 5.5 to 7.5), preferably with Tris or acetate buffer.

It is also preferred to isolate the proteinase K precipitate by centrifugation, preferably with 5,500 to 8,500 x *g* value from the AS solution.

Preferably, the proteinase K solution has a concentration of 20 mg/ml before ammonium sulfate is added.

Preferably, the added ammonium sulfate crystals are in powdered form. For example, ammonium sulfate crystals in powdered form that are commercially available in p.a. ("pro analysi") quality can be used. Such crystals are obtainable from suppliers of chemicals such as Mallinckrodt Baker.

It was surprisingly found that the solid form of proteinase K prepared according to the invention is not soluble in water in a considerable amount. Therefore, a further object of the invention is a preparation of a solid proteinase K preparation which is insoluble in water. However, the preparation is soluble in an aqueous solution containing 40 to 60% glycerol (w/v).

A further object of the invention is a method of solubilization of a solid preparation of proteinase K obtainable according to the invention by treating said preparation with an aqueous solution containing 40 to 60% glycerol (w/v).

The solid proteinase K can be in crystalline form, as precipitated or in powdered form.

Detailed description of the invention

The present invention provides a solid proteinase K preparation insoluble in water and obtainable by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, and isolating the precipitated proteinase K.

The precipitation of proteins such as proteinase K by the use of ammonium sulfate is well-known in the state of the art. It is a general method for protein separation and purification. The method is commonly known as salting-out. Ammonium sulfate has been the most widely used salt for such a method because of its high solubility. According to the state of the art, a saturated salt solution or powdered salt crystals are added to the protein mixture to bring up the salt concentration of the mixture.

The present invention provides a method for the preparation of proteinase K in a solid form by ammonium sulfate precipitation, characterized in that an aqueous solution of proteinase K is treated with a saturated aqueous solution of ammonium sulfate or with solid ammonium sulfate crystals under such conditions that proteinase K precipitates, characterized in that ammonium sulfate is added to said aqueous solution of proteinase K in that the ammonium sulfate concentration in said solution increases in an amount of 0.1 M to 0.2 M per 5 minutes up to a final concentration of 1.5 M to 2 M after 1 hour and the formed proteinase K precipitate is isolated.

Usually, while stirring, saturated ammonium sulfate solution is dropwise added to the protein solution until precipitation starts to form. Usually, protein precipitation will require 15 to 20 minutes up to about an hour to equilibrate. After the precipitation is performed, the mixture is centrifuged usually at about 10,000 *g* for 15 minutes and the precipitate is collected. Usually, ammonium sulfate precipitation is performed at a temperature between 0°C and 40°C. According to the invention it is preferred to work at about 4°C to 8°C.

During ammonium sulfate precipitation, the pH value is preferably about pH 7.5. After precipitation, centrifugation and washing with water, the solid proteinase K preparation can be either stored or dissolved in an aqueous glycerol solution. Solubilization is preferably performed by dialysis against such a glycerol-containing aqueous solution.

By the addition of ammonium sulfate, either in crystals or as a separate aqueous solution, the volume of the solution does not increase in a considerable amount. If ammonium sulfate is added as a separate aqueous solution, the volume increases usually by about 5%.

According to the invention it is possible to produce water-insoluble proteinase K according to the invention in every desired amount, e.g. from 1 mg or more to preferably up to 500 g or more.

Water-insoluble proteinase K crystals according to the invention can be washed using a buffer containing salts dissolved in water, or with pure water. Water-soluble impurities are thereby removed from the water-insoluble proteinase K. The washing step can be repeated. Therefore, another embodiment of the invention is a method for the purification of proteinase K, comprising the steps of (a) providing a solid proteinase K preparation insoluble in water and obtainable by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, and isolating the precipitated proteinase K; (b) resuspending the precipitated proteinase K of step (a) in water or an aequous buffer solution (pH 5.5 to 7.5); (c) isolating the solid proteinase K of step (b). It is preferred that the aequous buffer contains a buffer salt. Preferably, the buffer salt is a Tris salt. It is also preferred that the buffer is an acetate buffer. A preferred acetate buffer is Ca acetate buffer.

Crystallization of proteinase K is known in the state of the art in relation to X-ray crystallographic studies, e.g. by Pähler, A., EMBO J. 3 (1984) 1311-1314; Betzel, C., et al., Biochemistry 40 (2001) 3080-3088). However, such proteinase K crystals are prepared according to the usual methods for the production of X-ray crystals, which are cryo-cooling or microdialysis. Such crystals are not further purified and used as they are for X-ray investigations. In addition, such crystallization methods

are not useful for the production of large amounts of proteinase K crystals of high purity quality.

The present invention further provides a method for the solubilization of a solid form of proteinase K obtainable according to a method of the invention, that is to say obtainable by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, and isolating the precipitated proteinase K, characterized in that solubilization is performed by adding an aqueous solution of glycerol at a concentration of 40% to 60% (w/v).

Due to its insolubility in water the solid proteinase K preparation according to the invention can be used for situ solubilization in a reagent solution instead of adding a proteinase K solution. Therefore a further object of the invention is a method of inactivating a protein in a device containing said protein in an aqueous solution, solid proteinase K according to the invention and glycerol is added in an amount such that proteinase K is dissolved in an amount to inactivate said protein. Preferably the final concentration of glycerol is between 45% and 55%. Inactivation means that the protein shows no detectable characteristic activity like polymerase- or nuclease activity. Preferably the protein is a polymerase, desoxyribonnuclease, endonuclease or exonuclease. The amount of the protein is preferably between 14 to 20 mg/ml.

The following examples and references are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Examples:

Example 1

Crystallization of dissolved proteinase K

Buffer I consisting of 5 mM $CaCl_2$, 5 mM Ca-acetate, 1 mM EDTA, 10 mM TrisHCl pH 7.5 in water, is provided. Buffer I is cooled to a temperature between 4°C and 8°C. Commercially available proteinase K (EC-No. 3.4.21.14d) from Tritirachium album is provided as a lyophilizate. The lyophilizate is dissolved in Buffer I to result

in a protein concentration of 19-20 mg/ml. To this end, the lyophilizate is added slowly to the buffer while stirring. The buffer is stirred until the lyophilizate has dissolved completely. The pH of the mixture is monitored and re-adjusted, if necessary, to the value of pH 7.5 using HCl.

Subsequently, solid powdered ammonium sulfate salt crystals are added slowly and continuously to the mixture. The rate of adding ammonium sulfate crystals to the mixture is adjusted such that within 1 h the ammonium sulfate concentration in the mixture is brought up to 1.8 M (290 mg/ml). While adding ammonium sulfate to the mixture the mixture is stirred. Stirring is continued for 1 h when the concentration of ammonium sulfate is 1.8 M.

The precipitate portion, that is to say the crystallized matter of the resulting suspension is sedimented by centrifugation. The supernatant is removed.

Example 2

Washing of proteinase K crystals

The crystals obtained by the procedure of Example 1 are resuspended in Buffer I at a temperature between 4°C and 8°C. The volume in which the crystals are resuspended is about the same as the volume of the mixture of Example I. A smaller volume is also possible. The crystals are subsequently sedimented by centrifugation. The supernatant is removed.

Alternatively, the crystals obtained by the procedure of Example 1 are resuspended in water, at a temperature between 4°C and 8°C. The volume in which the crystals are resuspended is about the same as the volume of the mixture of Example I. A smaller volume is also possible. The crystals are subsequently sedimented by centrifugation. The supernatant is removed.

Example 3

Dissolving proteinase K crystals

Buffer II consisting of 5 mM $CaCl_2$, 5 mM Ca-acetate, 1 mM EDTA, 10 mM TrisHCl pH 7.5, 50% glycerol in water, is provided. Proteinase K crystals are

obtained by the procedure of Example 1 or Example 2. Crystals are resuspended in an aliquot of Buffer I. The suspension is transferred into dialysis tubes and dialyzed against Buffer II.

Alternatively, an amount of Buffer II is added to crystals obtained by the procedure of Example 1 or the procedure of Example 2.

Example 4

Enzymatic activity

As described in Example 1, proteinase K lyophilisate is dissolved in a volume of Buffer I at a final concentration of 19-20 mg/ml. Before adding ammonium sulfate salt a sample is taken from the solution and proteolytic activity is measured as activity per volume. Proteolytic activity is determined using hemoglobin as a substrate.

After the sedimentation step the proteolytic activity is determined in the supernatant. The sediment comprising the proteinase K crystals is washed three times with each washing step comprising resuspending the sediment in water or in Buffer I, and centrifuging the suspension. The volume of liquid in which the crystals are resuspended is equal to the volume of Buffer I in which the lyophilisate has been dissolved in the beginning. After each centrifugation step a sample is taken from the supernatant and the proteolytic activity in the supernatant is determined. After the last washing step the proteinase K crystals are dissolved in a volume of Buffer II which equals the volume of Buffer I in which the lyophilisate has been dissolved (see above). During this step the crystals dissolve completely and a clear solution is obtained. Again, the proteolytic activity is determined.

Tables I summarizes the proteolytic activities which are detected in the samples as given above. Activity per volume is given for each sample relative to the Aactivity per volume resulting from the dissolved lyophilisate in Buffer I.

Table I

Proteinase K lyophilisate dissolved in Buffer I	supernatant following precipitation with ammonium sulfate	supernatant after 1st washing step (water)	supernatant after 2nd washing step (water)	supernatant after 3rd washing step (water)	solution of proteinase K crystals after 3rd washing step
100%	2% – 5%	3% - 5 %	1% - 3%	not detectable	85% - 90%
Proteinase K lyophilisate dissolved in Buffer I	supernatant following precipitation with ammonium sulfate	supernatant after 1st washing step (Buffer I)	supernatant after 2nd washing step (Buffer I)	supernatant after 3rd washing step (Buffer I)	solution of proteinase K crystals after 3rd washing step
100%	2% – 5%	3% - 5 %	1% - 3%	not detectable	85% - 90%

List of References

Betzel, C., et al., Biochemistry 40 (2001) 3080-3088

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Ebeling, W., et al., Eur. J. Biochem. 47 (1974) 91-97

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CLAIMS:

1. Method for the purification of proteinase K, comprising the steps of

(a) providing a solid proteinase K preparation insoluble in water and obtained by adding to an aqueous solution of proteinase K ammonium sulfate in an amount of 0.1 to 0.2 M per 5 minutes up to a final amount of 1.5 to 2 M after 1 hour, so as to precipitate the proteinase K from solution;

(b) isolating the precipitated proteinase K obtained in step (a);

(c) resuspending the precipitated proteinase K of step (b) in water or an aqueous buffer solution;

(d) isolating the precipitated proteinase K obtained in step (c);

(e) resuspending the precipitated proteinase K of step (d) in water or an aqueous buffer solution; and

(f) isolating the precipitated proteinase K obtained in step (e), thereby obtaining purified proteinase K.

2. The method of claim 1, further comprising the steps of

(g) resuspending the precipitated proteinase K of step (f) in water or an aqueous buffer solution; and

(h) isolating the precipitated proteinase K obtained in step (g).

3. The method of claim 1 or 2, wherein in step (a) the aqueous solution of proteinase K comprises Tris and calcium acetate.

4. The method of any one of claims 1 to 3, wherein no proteinase K is measurably detectable in the supernatant when purified proteinase K of step (f) is contacted with water or an aqueous buffer solution.

5. Method for the solubilization of a preparation of proteinase K in solid form prepared in accordance with the method of claim 1, wherein said solubilization is performed by adding an aqueous solution of glycerol at a concentration of 40% to 60% (w/v) to the preparation of proteinase K obtained in step (f), thereby obtaining solubilization of a preparation of proteinase K in solid form.