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Description

The invention relates to a catalytically effective protein. It catalyses the transfer of γ-glutamylcysteine and/or γ-glutamylcysteine-S-derivatives.

The object of the invention is γ-glutamylcysteine transferase.

The enzyme is characterised by the following parameters:

Molecular weight 95,000 ± 10%, determined by gel filtration; enzyme present naturally as a dimeric protein (molecular weight in each case 47,000 ± 10%)
Optimum temperature 45°C
Optimum pH = 8.0
K_M value at 30°C and pH = 7.8 for glutathione 6.8 mMol and for glutathione-S-dimer 1.0 mMol
complete inhibition in the presence of ethylenediamine tetraacetic acid (EDTA), reversible after removal of EDTA and addition of heavy-metal ions
cofactors: heavy-metal ions

According to the invention, heavy metals are metals which have a density greater than the density of iron.
In particular, examples of such heavy-metal ions are the cations of the metals lead, tin, bismuth, titanium, manganese, cobalt, nickel, copper, silver, gold, platinum, zinc, cadmium, mercury, uranium, arsenic, selenium.

γ-glutamylcysteine transferase is obtainable by extraction of vegetable material. In principle, both the lower and the higher plants are suitable vegetable material, since the enzyme of the invention is an enzyme of primary metabolism. Examples of vegetable material are

Rauwolfia serpentina (Apocynaceae)
Asclepias syriaca (Asclepiadaceae)
Berberis stolonifera (Berberidaceae)
Beta vulgaris (Chenopodiaceae)

Silene cucubalus (Caryophyllaceae)
Helianthus annuus (Compositae)
Dioscorea composita (Dioscoreaceae)
Fumaria capreolata (Fumariaceae)
Agrostis tenuis (Graminaceae)
Iris pallida (Iridaceae)
Crotalaria cobalticola, Phaseolus vulgaris (Leguminosae)
Cissampelos mucronata (Menispermaceae)
Glaucium flavum (Papaveraceae)
Coptis japonica (Ranunculaceae)
Rosa canina (Rosaceae)
Morinda citrifolia (Rubiaceae)
Nicotiana tabacum (Solanaceae)
Viola calaminaria (Violaceae)

and:

Euglena gracilis, Fragillaria crotonensis, Navicula pelliculosa, Bumilleriopsis filiformis, Chlamydomonas reinhardii, Chlorella fusca, Monoraphidium minutum, Scenedesmus acutiformis, Stichococcus bacillaris, Sargassum muticum, Porphyridium cruentum (Phycophyta)
and
Amylostereum chailetti, Sarcosypha austriaca, Schizosaccharomyces pombe, Schizosaccharomyces versatilus, Sporobolomyces holsaticus (Mycophyta).

The higher plants Silene cucubalus and Crotalana cobalticola, and Schizosaccharomyces pombe are preferably used as fungus.

Cell cultures of the said plants are preferably used as vegetable material. However, the differentiated plants or plant parts including the roots can also be used to obtain the enzyme of the invention.

The extraction of vegetable material is carried out according to methods which have already been used to extract enzymes from plants. Usually, the cell structure of the plants or of the corresponding cell cultures is first destroyed. To this end, the vegetable material is advisably frozen, e.g. at the temperature of liquid nitrogen, where appropriate crushed, and finally taken up in an aqueous preparation of pH 6 to pH 10. In particular, buffer solutions suitable

for the said pH range, e.g. phosphate buffer, are used as aqueous preparations. Where appropriate, after separating non-decomposed vegetable material, the proteins can optionally be concentrated by precipitation thereof and subsequent gel filtration. The proteins are precipitated by adding electrolyte (in particular ammonium sulphate). The precipitated proteins are subsequently taken up in an aqueous buffer solution (pH = 6 to 10) and are subjected to gel filtration. The gel filtration achieves both desalination and separation of the existing protein mixture according to molecular size. The part of the eluate from the gel filtration containing the proteins of the molecular weight > 40,000 is used for further processing. The subsequent cleaning steps include the further chromatographic separation of the protein mixture on a hydrophobic column matrix (e.g. phenyl sepharose, Pharmacia, Freiburg), hydroxyapatite (Biorad) and ion exchangers (e.g. diethyl aminoethyl cellulose). The eluate is always selected using enzyme activity checks. If desired, other or additional separating methods can be used, such as electrophoresis, isoelectric focussing and the like.

The above-described processing procedures are carried out in the aqueous system, in particular in buffer solutions of pH 6 to 10, in particular pH 7 to 9, at temperatures of preferably 4 °C to 15 °C.

The process of the invention produces aqueous or where appropriate freeze-dried preparations, exhibiting enzymatic activity corresponding to a content of γ-glutamylcysteine transferase.

The invention therefore aims to provide enzymatically active compositions containing γ-glutamylcysteine transferase.

The enzyme of the invention can often be used advantageously in the immobilised state. To this end, the enzyme is physisorbed or chemisorbed [sic] onto carrier materials in a known manner. Examples of carrier materials are alginates, agaroses comprising functional groups, cellulose, polyacrylates (with e.g. oxiran groups), glass materials, silicates.

The enzyme of the invention catalyses the transfer of γ-glutamylcysteine and/or γ-glutamylcysteine-S-derivatives.

The invention therefore also aims to provide a process for enzymatic polymerisation, which is characterised in that compounds of the general formula I

$H_2N\text{-}CH(COOH)\text{-}(CH_2)_2\text{-}C(O)\text{-}NH\text{-}CH(CH_2\text{-}SR^1)\text{-}C(O)\text{-}X$,

wherein

R^1 denotes a hydrogen fragment, alkyl fragment or aryl fragment, and

X denotes a grouping of the general formula

NHR^2,

with R^2 equal to the hydrogen fragment, alkyl fragment or aryl fragment, or $NH\text{-}CH(R^3)\text{-}COOH$,

with the proviso that R^3 represents the appropriate fragment of all naturally occurring amino acids of the formula $NH_2CH(R^3)COOH$,

are converted in the presence of γ-glutamylcysteine transferase.

R^1 preferably denotes a hydrogen fragment, 2,4-dinitrophenyl fragment or dimer fragment,
R^2 preferably denotes a hydrogen fragment, carboxyethyl fragment, 4-nitrophenyl fragment or naphthyl fragment, and
R^3 preferably denotes a hydrogen fragment, hydoxyl fragment, methyl fragment and benzyl fragment.

Examples of compounds to be polymerised in accordance with the invention are glutathione, homoglutathione and γ-glutamylcysteineylamide.

The process of the invention yields polymers of the general formula II

$[H_2N\text{-}CH(COOH)\text{-}(CH_2)_2\text{-}C(O)\text{-}NH\text{-}CH(CH_2\text{-}SR^1)\text{-}C(O)]n\text{-}X$,

wherein the degree of polymerisation n lies within the range of 2 to 20.

Examples of such polymers are phytochelatin with R^1=H and $X=NHCH_2COOH$; homo-phytochelatin with R^1=H and $X=NHCH_2CH_2OH$, poly-(glutamylcysteine) with R^1=H and X=OH and poly-(glutamylcysteine) amide with R^1=H and $X=NH_2$.

The reaction temperatures are preferably 10 to 70 °C, in particular 25 to 50 °C.

An aqueous preparation of substances of the general formula I of pH 6 to 10, in particular 8.0, is preferably mixed and stirred with an aqueous enzyme preparation or a preparation in which the enzyme is present in the immobilised state, preferably in the presence of the afore-mentioned heavy-metal ions in a concentration of 0.1 μ mol/l to 10 mmol/l, in particular 10 to 500 μ mol/l.

The quantity of enzyme used, relative to the quantity of substrate, is in itself uncritical. It is adjusted according to the conversion of product required. Usually, enzyme quantities in the range of 1 to 50 g, in particular 15 to 20 g, are used per 1 mol of substrate to be used.

The reaction is brought about e.g. by high-pressure chromatography with underivatised or derivatised reaction products.

The conversion products in accordance with the general formula II are used for heavy-metal detoxification.

Example 1

Production of γ-glutamylcysteine transferase

500 g of a cell culture of Silene cucubalus frozen with liquid nitrogen were stirred with 10mM of mercaptoethanol for 20 minutes in 250 ml of an aqueous trihydroxymethylaminomethane-HCl-buffer solution (50 millimolar, pH = 8.5). The resulting cell homogenate was subsequently filtered off and centrifuged. 86 g/l of solid ammonium sulphate were added to the supernatant liquid. The resulting protein precipitation was centrifuged off and the proteins of the supernatant liquid were bonded to a phenyl sepharose column (2.5 x 20 cm, Pharmacia, Freiburg). The column was washed with 0.2 l ammonium sulphate solution (10 mM tris-HCl, pH = 7.8, 86g ammonium sulphate per litre, 10 mM mercaptoethanol), and the subsequently bonded protein was eluted with a solution of the composition 10mM tris-HCl, 10 mM mercaptoethanol and 10% ethylene glycol (pH = 7.8).
The eluate was pumped onto a hydroxyapatite column (1.5 x 11, 5cm; Biorad, Munich), which was equilibrated with a solution of 10 mM potassium phosphate buffer, pH = 7.8, 10 mM mercaptoethanol, 10 mM NaCl and 0.5 mM $MgCl_2$. The column was washed with 100 ml of the said buffer, and the

γ-glutamylcysteine transferase was subsequently eluted by a stepped gradient of the composition 20 to 100 mM potassium phosphate and 10 mM mercaptoethanol. The active fraction of this elution was applied to an ion exchanger ((anion) MonoQ, Pharmacia, Freiburg), after the potassium phosphate had been thinned to at least 1/10 of the initial concentration by ultrafiltration (Amicon, Düren) and washing the residue with a buffer of 20 mM tris-HCl buffer, pH = 8.5, 10 mM NaCl, 10 mM mercaptoethanol and 0.5 mM $MgCl_2$. It was eluted in the aforementioned buffer with a Na Cl gradient of 10-350mM NaCl. The active fraction of this elution was adjusted to 10 % w/w ammonium sulphate by addition of a 50 % ammonium sulphate solution in 10 mM tris-HCl, pH = 7.8, was pumped onto a phenyl suparose column (Pharmacia, Freiburg) and eluted with a linear gradient, consisting of A 86 g ammonium sulphate per litre in 10 mM tris-HCl, 10 mM mercaptoethanol, 10 mM NaCl and 0.5 mM $MgCl_2$, and B 10 mM tris-HCl, 10 mM mercaptoethanol, 10 mM NaCl, 0.5 mM $MgCl_2$ and 10% ethylene glycol. The active fraction of this elution was subjected to gel filtration on SW 3000 (LKB, now Pharmacia, Freiburg) in 50 mM β-alanine, 10 mM histidine, pH 6.6, 10 mM mercaptoethanol and 0.5 mM $MgCl_2$. The active fraction exhibited an activity of 1 ncat/mg protein. The yield was 10%.

Alternatively, the following cleaning process can be used after the hydroxyapatite column:

The active fractions were mixed with 10% glycerin, applied to a Aca 34-column (Pharmacia, Freiburg) and eluted with 10 mM tris-HCl buffer pH 7.8 and 10mM mercaptoethanol. The active fractions were charged onto an anion exchanger QAE FF (Pharmacia, Freiburg) and eluted with a KCl gradient of 150-400 mM KCl. The active fractions were adjusted to 10 % w/w ammonium sulphate by addition of pulverised ammonium sulphate, pumped onto a phenyl superose column (Pharmacia, Freiburg) and eluted with a linear gradient, consisting of A 85 g ammonium sulphate per litre in 10 mM tris-HCl buffer and 10 mM mercaptoethanol as well as B 10 mM tris-HCl buffer and 10 mM mercaptoethanol. The active fractions were dialysed over 10 L potassium phosphate buffer pH 8.0 (25mM) and subsequently applied to a chelating sepharose column (Pharmacia, Freiburg), which was charged with Zn^{2+}. Elution was carried out using 50 mM imidazole in 25mM potassium phosphate buffer pH 8.0. The active fraction exhibited a specific activity of 1 ncat/mg protein.

Example 2

Production of immobilised γ-glutamylcysteine transferase

6 ml of an enzyme preparation of γ-glutamylcysteine transferase in an aqueous tris-hydroxy-methylamine/HCl buffer solution (10 mM mercaptoethanol, 10 mM NaCl, 0.1 molar, pH = 7.8) with an enzyme activity of 1.2 ncat/mg and a protein content of 1.02 mg/ml were introduced into 50 ml of a 5 % w/w aqueous alginate solution under agitation. The enzyme-containing alginate solution was subsequently dripped into 500 ml of a 0.1 molar aqueous calcium chloride solution under agitation at 5 °C.
This yielded a spherical product of immobilised enzyme on calcium alginate, which was subsequently washed with the afore-mentioned buffer. The preparation exhibited an enzyme activity of 0.32 ncat/mg protein.

Example 3

Enzymatic polymerisation of glutathione

50 ml of an enzyme preparation of γ-glutamylcysteine transferase in an aqueous solution of tris-HCl (0.1 molar, pH 7.8), 15 mM glutathione, 10 mM NaCl with an enzyme activity of 1.1 ncat/mg protein and a protein content of 0.26 mg/ml were mixed continuously with 20 µmol of a 10 mM Cd^{2+} solution for one hour.
After an hour, 15 mg phytochelatins with a Cd^{2+} content of 18.2 % w/w were obtained. The Cd^{2+} component was removed by precipitation with gaseous hydrogen sulphide.

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Claims

1. A transferase enzyme which catalyses the transfer of the γ-glutamylcysteine fragment from glutathione, to form glutathione polymers (phytochelatins).

2. An enzyme according to Claim 1, characterised by the following parameters:
   a) molecular weight 95,000 ± 10%, determined by gel filtration; enzyme present as dimeric protein (monomer 47,000 ± 10%)
   b) optimum temperature 45°C
   c) optimum pH = 8.0
   d) K_M value at 30°C and pH = 7.8 for glutathione 6.8 mMol and for glutathione-S-dimer 1.0 mMol
   e) complete inhibition in the presence of ethylenediamine tetraacetic acid (EDTA), reversible after removal of EDTA and addition of heavy-metal ions
   f) cofactors: heavy-metal ions

3. An enzyme according to Claim 1, obtainable by extraction of vegetable material from the family of Apocynaceae, Asclepiadaceae, Berberidaceae, Chenopodiaceae, Caryophyllaceae, Compositae, Dioscoreaceae, Fumariaceae, Graminaceae, Iridaceae, Leguminosae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae, Solanaceae, Violaceae, and by extraction of the tissue of algae (phycophyta) and fungi (mycophyta).

4. Enzymatically active compositions containing the enzyme according to Claims 1-3.

5. An immobilized enzyme according to Claims 1-3.

6. A process for the production of the enzyme according to Claims 1-3, characterised in that vegetable material is extracted from the family of Apocynaceae, Asclepiadaceae, Berberidaceae, Chenopodiaceae, Caryophyllaceae, Compositae, Dioscoreaceae, Fumariaceae, Graminaceae, Iridaceae, Leguminosae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae, Solanaceae, Violaceae, and by extraction of the tissue of algae (phycophyta) and fungi (mycophyta).

* C L M S *

7. A process for enzymatic polymerisation, characterised in that compounds of the general formula I
H_2N-CH(COOH)-$(CH_2)_2$-C(O)-NH-CH(CH_2-SR^1)-C(O)-X,
wherein
R^1 denotes the hydrogen fragment, alkyl fragment or aryl fragment, and
X denotes a grouping of the general formula NHR^2, with R^2 equal to the hydrogen fragment, alkyl fragment or aryl fragment, or NH-CH(R^3)-COOH,
with the proviso that R^3 represents the appropriate fragment of all naturally occurring amino acids of the formula $NH_2CH(R^3)COOH$,
are converted in the presence of the enzyme according to Claims 1-3.