

This invention relates to a enzymatically obtained oligomer of L-amino acid, a process for its preparation and its uses.

More particularly the invention relates to an
5 enzymatically obtained oligomer of L-amino acid containing residues of L-methionine and consisting essentially of oligomers having a chain length of from 5 to 10 residues with an average length of 8. The invention also relates to a process for the preparation of this oligomer and to
10 its use either as an L-methionine substitute in the fortification of food diets or as an intermediate product in the preparation of L-methionine from DL-methionine.

Certain food ingredients which are naturally poor in sulphur-containing amino acids, such as soya proteins,
15 leaf proteins and microbial proteins, may be or have to be fortified with methionine, particularly for dietetic or infantile applications.

DL-methionine, which is produced by chemical synthesis on a scale of 100,000 t per year, is consumed above all by
20 cattle and poultry because it cannot be reliably used in foods for human consumption.

So far as human beings are concerned, only L-methionine is being considered for use at the present time. L-methionine is produced from DL-methionine on a scale of from
25 50 to 100 t per year by enzymatic separation of the two enantiomers. Because of this, L-methionine is approximately 10 times more expensive than DL-methionine.

Oligomers of methionine, principally the dimer, have been chemically synthesised:

- by the reaction of N-protected methionine, such as N-carbobenzoxy methionine, with the ethyl ester of methionine in the presence of a coupling agent, such as cyclohexyl carbodiimide, and cleavage of the protective group;
- from N-carboxy anhydride or Leuch's anhydrides in the presence of free amino acid, in aqueous medium, in the absence of heat and at a pH-value of 10 (Hirschmann method).

These methods are expensive and use unstable products or coupling reactants of which the purity has to be guaranteed.

In addition, poly-L-methionines of high molecular weight have been obtained from N-carboxy anhydrides by polymerisation in anhydrous medium. However, this method also involves the use of Leuch's anhydrides which are difficult to handle.

Accordingly, these methods have never been used in the preparation of food additives on account of their high cost and the guarantee of purity required in the interests of food hygiene.

It is therefore easy to appreciate the advantage there would be in having either a safe substitute for L-methionine, releasing it under ingestion conditions, or L-methionine available at a fairly moderate price.

The present invention meets precisely these two objectives. It provides an oligomer of L-amino acid

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containing residues of L-methionine consisting essentially of oligomers having a chain length of from 5 to 10 residues with an average length of 8 and having an L-methionine content amounting to from 60 to 90% by weight. In one
5 embodiment, the oligomer is a homo-oligomer consisting of residues of L-methionine. An oligomer such as this is insoluble in water and aqueous buffers and soluble in dimethyl sulphoxide (DMSO); its total nitrogen content amounts to from 9.5 to 10.5% and its specific rotation
10 $[\alpha]_D^{25}$ lies between -12 and -14° (DMSO).

The expression "insoluble in water" is understood to mean that, in vitro, the oligo-L-methionine does not dissolve significantly in water. This does not in any way pre-judge (rather on the contrary) its solubility in vivo in aqueous
15 media and biological fluids.

In one variant, the oligomer is a hetero-oligomer, i.e. in addition to the methionine residues, it contains residues of other amino acids. An oligomer such as this is soluble for example in aqueous media providing residues
20 of polar amino acids, such as glutamic acid or aspartic acid, are introduced therein.

Alternatively, it may be more digestible with respect to pancreatic enzymes than the homo-oligomer of L-methionine, for example through the introduction of residues of aromatic
25 amino acids, such as tryptophan, phenylalanine or tyrosine, through the creation of additional cleavage sites for the pancreatic proteases, which facilitates enzymatic hydrolysis.

In addition, the oligomer releases the L-methionine under acid pH-conditions such as are encountered in the stomach for example. It will therefore be appreciated that, through the oligo-L-methionine, it is possible to obtain L-methionine from DL-methionine, as described hereinafter in the disclosure of the invention. The process as a whole, although involving enzymatic intervention, is better suited to the preparation of L-methionine than the enzymatic resolution process mentioned above.

The present invention also provides a process for the preparation of the L-amino acid oligomer, which comprises reacting an ester of methionine and, optionally, of one or more other amino acids with a thiol-protease in an aqueous medium having a pH-value of from 5 to 7 at a temperature where the thiol-protease is active, and thereafter isolating the L-amino acid oligomer formed from the reaction medium.

It is pointed out that the starting methionine may be either L-methionine or DL-methionine. In the latter case, the reaction medium contains unreacted D-methionine in the form of its ester.

Preparation of the methionine ester, preferably the methyl or ethyl ester, is a simple, uncomplicated preliminary step. The operation involved may be carried out very easily by reacting methionine with the corresponding alcohol in the presence of thionyl chloride (SOCl_2).

In the case of a hetero-oligomer, it is sufficient to

add the required quantity of amino acid(s) in the form of its/their ester(s), preferably the methyl or ethyl ester(s), prepared as described above to the reaction mixture consisting of the methionine ester and the thiol-protease.

5 It is possible to use for example the ethyl esters of DL or L-tryptophan, of DL-tyrosine, α , γ -diethyl glutamate. Where a DL-amino acid is used to start with, an L-residue will be obtained as indicated above for methionine.

10 The thiol-protease used is preferably papain, ficin, or bromelin which are inexpensive and abundant enzymes. In their case, the reaction is carried out at a temperature in the range from 30°C to 50°C and preferably at a temperature of the order of 37°C.

15 The aqueous reaction medium used may be quite simply water. In that case, the pH-value should be monitored to ensure that it remains within the limits indicated above, preferably constant. As the enzymatic reaction progresses, an alkaline agent, such as 2N sodium hydroxide for example, will be added. Another possibility is to use a buffer
20 solution as the aqueous medium, for example a buffer solution of sodium salts of carboxylic acids, Na citrate, Na succinate or Na acetate.

The pH-value of the aqueous medium is the determining factor for the enzymatic reaction and should be strictly
25 kept from pH 5 to 7. Advantageously, this pH-value is selected at around 6.5 where the reaction is carried out in water and at around 5.5 where the reaction is carried

out in a buffer.

On completion of the enzymatic reaction by which the oligomer is formed, the originally clear reaction solution has changed to a milky suspension. The enzyme is then
5 advantageously deactivated, for example by brief heating to 90°C, after which the solid phase forming the required oligomer is separated from the liquid phase. In addition to impurities and secondary reaction products, the reaction medium contains in solution unreacted D-amino acid in the
10 form of its ester (methyl or ethyl ester) where the starting amino acid is in the DL form.

To improve the purity of the oligomers, the solid phase is washed liberally with water. Separation of the phases may be facilitated by centrifuging.

15 Purification may also be carried out by dialysis or by ultrafiltration after the solid phase has been re-suspended in water. It is preferred to apply diafiltration, i.e. ultrafiltration with dilution of the retentate. In that case, dilution may be carried out continuously keeping the
20 level constant or even in batches by ultrafiltration without dilution, followed by re-adjustment to the original level before the next ultrafiltration step.

This procedure gives the L-amino acid oligomer of which the characteristics are mentioned above and of which the
25 characterisation means will be indicated in the Examples. In the presence of pepsin in acid medium, the oligomer according to the invention is hydrolysed, giving for the

most part L-methionine accompanied by di-L-methionine and tri-L-methionine. It is pointed out that, in the case of a homo-oligomer, the oligo-L-methionine may perform the function of L-methionine proper in the fortification of food additives which are poor in methionine.

Under conditions of high acidity, the oligo-L-methionine is cleaved into L-methionine with a negligible degree of racemisation. Preparation of the oligo-L-methionine from DL-methionine followed by its hydrolysis in acid medium, thus represents an elegant way of preparing L-methionine from its racemic compound.

In a first embodiment of the process according to the invention, a hydrochloride of a methionine ester is dissolved in water in a concentration of preferably from 1 to 2 M. The pH is kept constant at 6.5 throughout the reaction by adding 2N NaOH. Where the methionine used is DL-methionine, its concentration is of course two times higher than where the starting methionine is L-methionine.

The enzyme, mainly papain, bomelin or ficin, is added in a quantity of 2% by weight, preferably in two additions of 1% each. On completion of the reaction, i.e. after about 6 hours, a milky suspension has formed and is briefly heated to 90°C to deactivate the enzyme which it contains. The two phases (solid and liquid) are separated, after which the solid phase - representing the oligo-L-methionine - is washed by diafiltration.

In a second embodiment, the reaction is carried out at

a pH-value around 5.5. in a buffer of sodium citrate, succinate or acetate.

The same experimental procedure as described above may be applied in the case of a hetero-oligomer by dissolving the various hydrochlorides of the amino acids in question in the proportions selected in water in the first stage.

The following Examples illustrate the invention and describe the methods used to characterise the oligo-L-methionine. In these Examples, the proportions and percentages quoted represent values by weight.

EXAMPLE 1

Hydrochloride of ethyl ester of L-methionine is conventionally prepared by the action of SOCl_2 on a solution of L-methionine in ethanol.

The hydrochloride thus formed is then dissolved in water in a quantity of 300 mg per ml (1.4 M). The solution is placed in a pH-stat so as to keep the pH-value constant at 6.5 during the reaction by the addition of 2N NaOH.

A 0.1 M aqueous solution of papain in L-cysteine is then added in a quantity of 1% by weight of enzyme. 1 hour later, another 1% of the enzyme is added in the same way as before.

After a reaction time of 6 hours at 37°C at the pH kept strictly constant at 6.5, the originally clear reaction medium has changed into a milky suspension. This suspension is then briefly heated to 90°C to deactivate the enzyme, followed by separation of the solid phase which is

washed liberally with water.

The solid phase thus separated, which represents the crude oligo-L-methionine, is re-suspended in 20 times its weight of water and then introduced into an ultrafiltration cell equipped with an Amicon UM 10 membrane under a nitrogen pressure of 2.5 atms and at a temperature of 4°C (Amicon is a Trade Mark). Ultrafiltration is continued until the volume has been reduced to one twentieth of the initial volume, after which the initial volume is restored by the addition of water and the process is repeated. When about 100 volumes of filtrates have been recovered, ultrafiltration is stopped and the retentate is dried by freeze drying.

This retentate represents the purified oligo-L-methionine.

Characterisation of the oligo-L-methionine

I. The infra-red (I.R.) spectrum in the solid state in a concentration of 1% in KBr shows the following characteristic bands:

3230 $\text{cm}^{-1}(\text{s})$: N-H stretching characteristic of a β structure.

1710 $\text{cm}^{-1}(\text{w})$: C=O : terminal carboxyl groups

1620 $\text{cm}^{-1}(\text{s})$: C=O stretching: amide band I

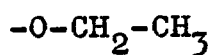
1520 $\text{cm}^{-1}(\text{s})$: N-H deformation: amide band II

700 $\text{cm}^{-1}(\text{m})$: vibration outside the plane: amide band V.

II. The nuclear magnetic resonance (N.M.R.) spectrum of the proton of the crude oligo-L-methionine in dimethyl sulphoxide (DMSO-d₆) at 80 MHz shows the following characteristic signals:

8.2 ppm : multiplet (2): terminal amino group $\text{H}_2\text{N}-$

4.4 ppm : multiplet (approx. 10 x 2): methylene protons



2.4 ppm : multiplet }
1.8 ppm : shoulder } α , β and γ protons
2.0 ppm : singlet (approx. 10 x 2): thioether protons
-S-CH₂

5 1.2 ppm : triplet (3): methyl protons -O-CH₂-CH₂.

III. An elution profile on fine Sephadex G25 resin in dimethyl sulphoxide (DMSO) shows that the oligo-L-methionine is relatively homogeneous, the main peak corresponding - according to the elution volumes and the working conditions -
10 to the octamer (Sephadex is a Trade Mark).

IV. Other characterisation data are given below:

- insoluble in water and aqueous buffers,
- soluble in dimethyl sulphoxide (DMSO),
- total nitrogen content between 9.5 and 10.5%, specific
15 rotation $[\alpha]_D^{25} = -12$ to -14° (DMSO),
- circular dichroism $\theta = 1922^\circ$, two negative bands at 230 and 195 nm,
- elemental analysis compared with the theoretical (calculated)
20 elemental analyses for the oligomers containing from 6 to 10 methionine residues after cleavage of the ester group by hydrolysis: % C = 45.22; % H = 7.62; % N = 10.58, which corresponds to an average number of residues of 8,
- methionine content between 78 and 90%.

EXAMPLE 2

25 The procedure described in Example 1 is repeated using DL-methionine instead of L-methionine in twice the concentration.

After diafiltration, the same oligo-L-methionine as in Example 1 is obtained, as shown by the physical properties and the IR and NMR-spectra.

EXAMPLE 3

5 The procedure described in Example 1 is repeated using L-methionine, but with ficin instead of papain as the enzyme.

The oligo-L-methionine obtained is similar to that of Example 1.

10 EXAMPLE 4

The methyl ester of methionine is prepared by the action of SOCl_2 on a solution of methionine in methanol, and isolated in the form of its hydrochloride.

15 The procedure described in Example 1 is then repeated using this methyl ester instead of the ethyl ester of that Example in corresponding proportions.

An oligo-L-methionine similar to that of Example 1 is thus obtained.

EXAMPLE 5

20 The procedure described in Example 1 is modified by using as the reaction medium an aqueous buffer in the form of a 1 M solution of sodium citrate having a pH of 5.5. In this case, the pH remains constant throughout the reaction so that no NaOH has to be added. For the rest, the procedure
25 is as described in Example 1.

An oligo-L-methionine similar to that described in Example 1 is thus obtained.

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EXAMPLE 6

The procedure described in Example 2 starting with DL-methionine is repeated using a 1 M sodium acetate buffer of pH 5.5 in accordance with the preceding Example. Once again, the concentration of the DL-methionine is twice that of the L-methionine on its own.

An oligo-L-methionine similar to that described in the preceding Examples is thus obtained.

EXAMPLE 7

The oligo-L-methionine described in the preceding Examples is subjected to enzymatic digestion with pepsin from pigs in a quantity of 2% by weight. To this end, the oligo-L-methionine is suspended for 24 hours in a quantity of 2% by weight, at pH 2 and at a temperature of 37°C. Over this period, 85% of the oligo-L-methionine is converted into soluble products. These soluble products consist essentially of free L-methionine (accompanied by traces of the ethyl ester of L-methionine) and of di-L-methionine and tri-L-methionine.

EXAMPLE 8

1 g of the oligo-L-methionine described in Examples 1 to 6 is subjected to acid hydrolysis under nitrogen in approximately 500 ml of 6N hydrochloric acid for about 20 hours at 105 to 110°C. The acid is then eliminated by distillation under reduced pressure and the hydrolysate is taken up in water. The chromatographically (thin-layer) and electrophoretically pure L-methionine is identified therein along with approximately 1 to 2.5% of D-methionine.

The small quantity of D-methionine present may be attributable to the presence of residual D-methionine in the oligomer or to racemisation caused by chemical hydrolysis. The isolated and recrystallised amino acid has a specific rotation $[\alpha]_D^{25}$ of +22 to +23° (6N HCl). By comparison commercial analytical quality L-methionine has a rotation $[\alpha]_D^{25}$ of + 23.7° $\pm$ 0.5°.



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CLAIMS

1. An enzymatically obtained oligomer of L-amino acid, containing residues of L-methionine, consisting essentially of oligomers having a chain length of from 5 to 10 residues with an average length of 8 and having an L-methionine content amounting to from 60 to 90% by weight.
2. An oligomer as claimed in Claim 1, containing from 78 to 90% by weight of L-methionine, which is insoluble in water and aqueous buffers and soluble in dimethyl sulphoxide (DMSO), having a total nitrogen content amounting to from 9.5 to 10.5% by weight and having a specific rotation $[\alpha]_D^{25}$ of from -12 to -14° (DMSO).
3. An oligomer as claimed in Claim 1, which contains residues of polar amino acids or aromatic amino acids.
4. An oligomer as claimed in Claim 3, wherein the polar amino acids are glutamic acid or aspartic acid, and the aromatic amino acids are tryptophan, phenyl alanine or tyrosine.
5. An enzymatically obtained oligomer of L-amino acid as claimed in claim 1 substantially as described with particular reference to any of Examples 1 to 6.
6. A process for preparing an oligomer of L-amino acid as claimed in any of Claims 1 to 5, which comprises reacting

an ester of methionine and, optionally, of one or more other amino acids, with a thiol-protease in an aqueous medium having a pH-value in the range from 5 to 7 at a temperature where the thiol-protease is active, and thereafter isolating the L-amino acid oligomer formed from the reaction medium.

7. A process as claimed in Claim 6, wherein the thiol-protease is deactivated before isolation of the L-amino acid oligomer.

8. A process as claimed in Claim 6 or 7, wherein the methionine is L-methionine or DL-methionine.

9. A process as claimed in any of Claims 6 to 8, wherein the other amino acid(s) is/are glutamic acid, L-tryptophane, DL-tryptophane or DL-tyrosine.

10. A process as claimed in any of Claims 6 to 9, wherein the ester of methionine and the other amino acid(s) is the methyl or ethyl ester.

11. A process as claimed in any of Claims 6 to 10, wherein the thiol-protease is papain, ficin or bromelin and the aqueous reaction medium is kept at a pH of from 5 to 7 by means of a pH-stat or is a buffer solution of a sodium salt of a carboxylic acid.

12. A process as claimed in any of Claims 6 to 11, wherein the L-amino acid oligomer is isolated by ultra-



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filtration with dilution of the retentate during the ultra-filtration process.

13. A process for preparing an oligomer of L-amino acid as claimed in claim 6 substantially as described with particular reference to any of Examples 1 to 6.

14. An oligomer of L-amino acid when prepared by a process as claimed in any of Claims 6 to 12.

15. A food poor in methionine having added thereto an oligomer of L-amino acid as claimed in any of Claims 1 to 5 or 14 to enhance its nutritive value.

16. A process for the production of L-methionine which comprises hydrolysing in acid medium an oligomer of L-amino acid as claimed in any of Claims 1 to 5 or 14 and prepared from DL-methionine.

17. L-methionine when prepared by a process as claimed in Claim 16.

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