METHOD FOR INACTIVATING PROTEASES BY PH CHANGE IN A LIQUID OBTAINED FROM A CELL CULTURE

BACKGROUND TO THE INVENTION

TECHNICAL FIELD

The invention is in the field of the manufacture of biopharmaceutical products. It relates in particular to improving the process for preparing biopharmaceutical products by the inactivation of proteolytically active enzymes in the cell-free cell culture supernatant.

BACKGROUND

Biomolecules such as proteins, polynucleotides, polysaccharides and the like are increasingly gaining commercial importance as medicines, as diagnostic agents, as additives to foods, detergents and the like, as research reagents and for many other applications. The need for such biomolecules can no longer normally be met - for example in the case of proteins - by isolating molecules from natural sources, but requires the use of biotechnological production methods.

The biotechnological preparation of proteins typically begins with the cloning of a DNA fragment into a suitable expression vector. After transfection of the expression vector into suitable prokaryotic or eukaryotic expression cells and subsequent selection of transfected cells the latter are cultivated in fermenters and the desired protein is expressed. Then the cells or the culture supernatant is or are harvested and the protein contained therein is worked up and purified.

It is known that proteases are present in the harvested liquid, e.g. in cell-free culture supernatant. Both biopharmaceuticals such as monoclonal antibodies or recombinant proteins as well as chromatography materials such as immobilised protein A can be very rapidly degraded or structurally damaged by proteases. This leads to compromises in the product quality (homogeneity, functionality) and, in chromatographic materials, to a reduction in the binding capacity, with consequent contamination of the bound product fractions. Particularly in serum-free cultivation and in highly productive cells the biopharmaceuticals produced are present in high relative concentrations and are thus

particularly prone to proteolytic damage to the molecular structure, leading to both a reduced yield and lower product quality.

Protein damage caused by proteases may occur even at neutral pHs, but extensive protein degradation may be observed particularly when the cell-free culture supernatant has to be adjusted to acidic pH levels for the purification process, for example, in order to create the desired binding conditions for the capture step, e.g. cation exchange chromatography (conditioning).

It is known that some proteases, e.g. digestive enzymes such as pepsin, are irreversibly inactivated by changes to the pH level (Z. Bohak, Purification and Characterization of Chicken Pepsinogen and Chicken Pepsin, Journal of Biological Chemistry 244 (17) (1969) 4633–4648; B. Turk, V. Turk, Lysosomes as Suicide Bags in Cell Death: Myth or Reality?, Journal of Biological Chemistry 284 (33) (2009) 21783–21787). The addition of protease inhibitors has also been proposed (A.J. Barrett, A. A. Kembhavi, M. A. Brown, H. Kirschke, C. G. Knight, M. Tamai and K. Hanada, L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L, Biochem. J. 201 (1982) 189–198). However, these are very expensive, toxic and difficult to eliminate from the product. Therefore they are not an option for the economic production of safe medicaments.

BRIEF SUMMARY OF THE INVENTION

The invention relates to a method of inactivating proteases by repeatedly changing the pH in the cell culture supernatant at the start of the process for the purification of biopharmaceuticals. Advantages of the invention are an improvement in product quality and product yield, and a longer life for chromatographic materials.

Surprisingly it has been found that the harvested cell-free fermentation supernatants of mammalian cell lines (e.g. CHO, "Chinese hamster ovary" cells) contain proteases that can be activated by changing to an acidic pH and can also be irreversibly inactivated in their activity at the optimum pH by subsequently changing the pH to the neutral range. Proteases that are active at neutral pH levels can also be irreversibly inactivated in their activity under neutral conditions by a change to acidic pH levels.

The present invention particularly relates to a process for inactivating proteases in liquids which are obtained from cell cultures, comprising the steps of:

(a) adjusting the pH of the liquid to 3 to 5, and then

(b) adjusting the pH of the liquid to 7 to 9.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Activation and inactivation of proteases from CCF by changing the pH twice.

Figure 2: Degradation of the model substrate interferon (IFN) at an acidic pH. 0.1 mg/ml interferon was incubated at 37°C with 10 % (v/v) cell culture supernatant (CCF) at pH 4 for 0 or 14 hours with and without a change in pH (lane 2 to 4) and separated by SDS-PAGE. The change in pH took place at 20°C with 5 minute pauses at pH 4 and at pH 7. IFN is degraded significantly less by the change in pH (lane 3) than without pH inactivation. After 14 hours IFN has been broken down completely (lane 4).

Layout of lanes:

1 – marker

2 – IFN (0.1 mg/mL) before incubation

3 – CCF + IFN pH 4 with change in pH, 14 h incubation

4 – CCF + IFN pH 4 without change in pH, 14 h incubation

Figure 3: Breakdown of the protein IFN by three hours' incubation with CCF, 10 % (v/v) at pH 4.0, analysis with RP-HPLC. After inactivation of the proteases by neutralisation and subsequent incubation with IFN at pH 4.0, after three hours 72 % of the IFN can still be detected by RP-HPLC, whereas at the same time, without inactivation, only 43 % of the IFN are still intact. The proteolytic activity can thus be reduced by half compared with a wild-type protein.

Figure 4: Fluorescence assay at an acid and neutral pH. Proteases in the CCF are active at pH 3.5 (●) and at pH 7 (▲). As a measurement of the proteolytic activity produced by

proteases present in the CCF at pH 3.5 and pH 7, the release of a fluorophore by cleaving a peptide substrate was measured.

Figure 5: Proteolytic activity of neutral proteases with and without a change in pH. Neutral proteases may be almost completely and irreversibly inactivated by acidification to pH < 5 and subsequent neutralisation. The measurement was carried out at pH 7 in each case, the release of a fluorophore by cleaving a peptide substrate was measured as a measurement of the proteolytic activity.

Figure 6: Proteolytic activity of acid proteases without and with a change in pH. The activation/inactivation of the proteases in the CCF was carried out by changing the pH analogously to Figure 1. Activated proteases are active at pH < 5 and cleave the substrate. Activated proteases which had been inactivated by a brief incubation at pH ≥ 7 exhibit a residual activity reduced to 35% at pH 3.7. The measurement was carried out at pH 3.7 in each case, the release of a fluorophore by cleaving a peptide substrate was measured as an indication of the proteolytic activity.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a process for inactivating proteases by repeatedly changing the pH of the cell culture supernatant at the start of the purification process. Advantages of the invention are an improvement in the product quality and yield as well as a lengthening of the life of chromatographic materials.

Surprisingly, it was found that the harvested cell-free fermentation supernatants of mammalian cell lines (e.g. CHO, "Chinese hamster ovary" cells) contain proteases that can be activated by changing to an acidic pH and can also be irreversibly inactivated in their activity at the optimum pH by subsequently changing the pH to the neutral range. Proteases that are active at neutral pH values can also be irreversibly inactivated in their activity under neutral conditions by a change to acidic pH levels.

In another aspect the invention relates to a process for reducing protein degradation in liquids which are obtained from cell cultures, comprising the steps of:

(c) adjusting the pH of the liquid to 3 to 5, and then

(d) adjusting the pH of the liquid to 7 to 9.

After acidification of the cell culture supernatant, activation of the proteases present obviously takes place, indicating the presence of originally lysosomal proteolytic enzymes (cathepsins). These proteases are involved in the breakdown of endocytic proteins, are ubiquitously expressed in all tissues as non-active proforms and are located intracellularly in endosomes. The maturation of these compartments to form lysosomes is accompanied by a dramatic lowering of the pH which leads to both autocatalytic and *in trans* activation of the lysosomal proteases. The secretion of cathepsins into the extracellular space is discussed chiefly in the context of the metastasisation of tumour tissue, and for some individual cathepsins secretion in cell culture has also been described.

The proteolytic activity at neutral pH values can be attributed on the one hand to secreted proteases and on the other hand to proteases originally located in the membrane, which are presumably separated from the cell membrane during the production process and continue to be active in solution.

Typically, in biopharmaceutical processes, cells are separated from the product-containing cell culture supernatant by centrifugation or filtration. The cell-free supernatant is then sterile filtered (max. 0.2 µM pore size) and diafiltered for rebuffering before the capture step. The inactivation by changing the pH twice can be carried out at the earliest immediately after the separation of the cell culture supernatant from the cells and used in any other subsequent process step.

When selecting the pH levels the product molecule and the technical equipment should not be damaged, and therefore pH levels < 3 should be avoided (chemical modification of the product protein and increased corrosion of steel containers), as well as pH > 9 (deamidation of asparagine and glutamine). The retention times at the respective pH values also depend on the stability of the product protein. The time span for the activation of proteases by acidification should be as short as possible, but advantageously at least 5 minutes (min). For example, retention times between 5 and 30 minutes are advantageous, preferably 5 to 15 minutes. With longer retention times for activation at acidic pH levels, there may be increased proteolytic breakdown of the target protein. The time span of the subsequent retention step for inactivation of the acid proteases at a

neutral pH is not critical and the pH can also be maintained over several process steps or varied again, as all neutrally active proteases have already been inactivated and no more proteolytic activity can be detected. Advantageous retention times for the neutralising step are 5 to 60 minutes, for example.

The adjustment to the respective target pH values may be carried out in solution by a one-time addition or titration of acids such as acetic acid or hydrochloric acid or lyes/bases such as sodium hydroxide solution or Tris, with stirring. In the acid step pH values of between 3 and 5 are advantageously selected, for example a pH of 3.5 to 4.5, preferably 4. For the subsequent inactivation of acid proteases by neutralisation, pH values of 7-9 have proved effective, preferably pH 7.4-8.5.

The invention may be carried out in a temperature range of 4°C- 37°C, preferably 15°C to 37°C, preferably 20-37°C. A preferred range for performing the invention is 20°C to 30°C.

The process for inactivating acid and neutral proteases by changing the pH twice was successfully carried out on cell-free culture supernatants of mammalian cells (CHO and NS0). The results can also be transferred to culture supernatants of other production organisms and can be used within the scope of the requirements of the product protein, particularly its pH stability, in the manufacture of various biopharmaceutical products.

The present invention makes use of purely physico- and biochemical methods. By changing the pH twice through different pH units (Figure 1) up to 75% of the acid protease activity and up to 90% of the neutral protease activity can be irreversibly eliminated. The protease activity can be detected using two detection methods, a) by the release of fluorescence after peptide cleaving and b) by the degradation of native protein substrates.

Proteases that are harmful to the product and equipment can be irreversibly inactivated during the production of biopharmaceutical medicaments by a quick and simple physicochemical method. The cell culture supernatant can be used in a variable manner as a result and may be obtained by a variety of purification techniques.

The addition of acids and lyes to tanks is very quick and easy to carry out and can also be scaled up for industrial use. After the inactivation of the proteases the cell culture

supernatant may be adapted to the purification processes in a very variable manner. Standing times or pH values are non-critical, by contrast with the conventional production processes.

Examples

Working example 1: Change in pH after harvesting, before the capture step

CHO cells are grown in the fed batch, final volume 80 L, for 11 days. The cell culture supernatant (CCF) is maintained at 20°C and separated from the cells using a throughflow disc centrifuge and sterile-filtered through a filter cascade. Then the pH value of the CCF is lowered to pH 4 by the addition of acetic acid. After the target pH has been reached it is maintained for 5-10 min, before the CCF is neutralised to pH 7.5 by the addition of sodium hydroxide solution. Before further processing (capture step) the CCF is ultra-/diafiltered through a 50 kD MWCO membrane in order to achieve suitable binding conditions for the capture step.

Working example 2: Change in pH directly after the separation of CCF and cells, before sterile filtration

CHO cells are grown in the fed batch, final volume 80 L, for 11 days. The cell culture supernatant (CCF) is separated from the cells using a throughflow disc centrifuge and maintained at 20°C. Then the pH value of the CCF is lowered to pH 4 by the addition of acetic acid with constant stirring. After the target pH has been reached it is maintained for 5-10 min, before the CCF is neutralised to pH 7.5 by the addition of sodium hydroxide solution. The treated CCF is then sterile-filtered through a filter cascade. Before further processing (capture step) the CCF is ultra-/diafiltered through a 50 kD MWCO membrane in order to achieve suitable binding conditions for the capture step.

Working example 3: Change in pH after rProtA capture step, before inactivation of the virus

CHO cells are grown in the fed batch, final volume 80 L, for 11 days. The cell culture supernatant (CCF) is separated from the cells using a throughflow disc centrifuge, sterile-filtered through a filter cascade and ultra-/diafiltered through a 50 kD MWCO membrane in order to achieve suitable binding conditions for the capture step, rProteinA affinity chromatography on PBS pH 7.5. A MabSelect chromatography column is charged with 32 mg of mAb per mL of column matrix and the antibody is eluted in a step with acetate buffer pH 3.5. The pH of the fraction containing the product is adjusted to pH 7.5 by the addition of 1 M Tris, with stirring, and the pH is maintained for 10 min at ambient temperature before suitable conditions for an acidic inactivation of the virus are selected.

Material and methods

Cell culture supernatant

Murine and CHO production cell lines optimised to the secretory production of therapeutic proteins are cultivated for a number of days in serum-free medium. The cell culture supernatant (CCF, cell free cell culture fluid) is separated by filtration or centrifugation from cells and insoluble constituents and after being adjusted to the respective pH it is used at 10-20 % (v/v) for the activity assays.

Adjustment of the pH value

The cell culture supernatants are acidified by the addition of acetic acid. The samples are immediately mixed and incubated for 5-10 minutes at the selected pH. Precipitating constituents are pelleted by centrifugation and discarded. The pH is raised by the addition of sodium hydroxide solution or 1 M Tris base.

Inhibition experiments for determining the protease classes

In order to inhibit individual protease classes, CCF is incubated with different commercial inhibitors and any remaining activity is then investigated in the activity assays. The concentration of inhibitor used is that recommended by the manufacturer.

Activity assays

Fluorescence assay, analysis by the release of a fluorophore

The substrates used for the kinetic and quantitative determination of the proteolytic activity are different peptide-fluorophore conjugates, the cleaving of which leads to the release of fluorescent dyes such as aminomethylcoumarin (AMC) or the elimination of the quenching effect by dinitrophenyl (Dnp) on N-methylaminobenzoyl-diaminopropionic acid (Nma). The increase in the fluorescence signal may be monitored photometrically at λ_{Ex}= 380 nm; λ_{Em} = 460 nm (AMC), λ_{Ex} = 340 nm; λ_{Em}= 460 nm in the Multilabel-Counter Victor[2, 3] (Perkin Elmer, Massachusetts, USA) (Fig. 3).

All the assays for kinetic and quantitative analyses are carried out at with saturation of the substrate at 0.2 mM Peptide-AMC, 10-20 µM Peptide-MCA or 5-10 µM DnP-Peptide-Nma in the presence of 10 - 20 % (v/v) CCF at pH 7 (100 mM Tris/HCl, 200 mM NaCl) and pH 3.5 (100 mM Na-acetate, 200 mM NaCl).

Degradation assay, analysis by PAGE and RP-HPLC

The substrate used for the qualitative analysis of proteolytic activity is a native protein of the interferon family (IFN). IFN is 22.5 kD in size and is present as a monomer in solution. For the degradation assay, 0.1 mg/ml IFN are incubated with 10 % (v/v) CCF at 37°C at pH 4 for up to 24 hours and detected by SDS-Page and silver staining according to Heukeshoven (Heukeshoven, J., Dernick, R., Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulphate gels. Electrophoresis, 9, (1988) 28-32.) or the degradation of the protein is determined by RP-HPLC as a measurement of proteolytic activity.

The RP-HPLC analysis is carried out on an HPLC apparatus made by Waters (Waters 2695 alliance) with a UV detector (Waters 2487 Dual Absorbance Detector) by means of a Vydac 214 TP-C_4 column by gradient elution of 0.2 % (v/v) TFA in water (solution A) to 0.15 % (v/v) TFA in acetonitrile (solution B) (Table 1).

Table 1: Elution gradient for a RP-HPLC-C4 column. The gradient runs from aqueous to organic solvent

time [min]	solution A [%]	solution B [%]
5.0	60	40
15.0	30	70
15.1	10	90
19.0	10	90
19.1	60	40
21.0	60	40

Results

The substrate cleaving by proteases from CCF has two peaks which are situated at acidic pH values pH < 5 and in the neutral range around pH 7. The activities of the respective proteases may be monitored by protein breakdown and the release of fluorescence after the cleaving of a fluorogenic peptide substrate (Fig. 2 and 4). Figure 2 shows the breakdown of IFN at pH 4 in the presence of 10 % (v/v) CCF. After only a few hours IFN is broken down under acidic conditions (lane 4), Figure 4 shows the course of the cleavage over time of the Dnp-peptide-Nma-substrate by 20 % (v/v) CCF after incubation at pH 3.5 and pH 7. The increasing fluorescence signal is a measurement of the cleavage of the peptide substrate. Activity is observed at both acidic and neutral pH values.

This activity can be suppressed by protease class-specific inhibitors, thereby showing that a number of protease classes are present in the CCF and can be divided into two groups: the acidically-active proteases which are active only at low pH levels, and the neutrally-active proteases which are active only at neutral pH levels. The acidically active ones have no activity at neutral pH values and the neutrally active ones have no activity at acidic pH values. Whereas the neutrally-active proteases are already active at the time of cell separation, the activation of the acidically active proteases in CCF does not take place until the reaction conditions are acidified.

Two-step change in the pH in order to inactivate neutral proteases

The activity of neutral proteases from untreated CCF may be determined at pH 7 in the fluorescence assay (Fig. 5, circles). If the CCF is subjected to a two-step change in pH from pH 7 to pH < 5 followed by neutralisation, virtually no further activity can be detected in the fluorescence assay at pH 7 (Fig. 5, triangles).

The brief acidification of the CCF and subsequent neutralisation lead to total and irreversible loss of the proteolytic activity of neutrally-active proteases.

Two-step change in the pH in order to inactivate acidic proteases

The proteolytic activity of the proteases activated by the acidification of the CCF may be detected at pH 3.5 in the fluorescence assay and in the protein degradation assay (Fig. 6, squares). However, this activity is not maintained if the CCF is neutralised after the acidification. If the reaction conditions of pH 3.5 that are optimal for acidic proteases are restored after the neutralisation, the activity of the acidic proteases that is measurable in the fluorescence assay is reduced by up to 65 % compared with the untreated CCF (Fig. 6, triangles).

The degradation of proteins is also reduced by the double change in the pH. The degradation of the model protein IFN is significantly reduced after the neutralisation step (Fig. 3, grey bars).

It was possible to reduce the breakdown of the native protein substrate IFN by 50% as a result of the repeated change in the pH (Fig. 3, quantification by RP-HPLC).

CLAIMS

1. Process for inactivating proteases in liquids that are obtained from cell cultures, comprising the steps of:
   (a) adjusting the pH of the liquid to 3 to 5, and then
   (b) adjusting the pH of the liquid to 7 to 9.

2. Process for reducing the protein degradation in liquids that are obtained from cell cultures, comprising the steps of
   (a) adjusting the pH of the liquid to 3 to 5, and then
   (b) adjusting the pH of the liquid to 7 to 9.

3. Process according to claim 1 or 2, characterised in that the pH in step (a) is in the range from 3.5 to 4.5.

4. Process according to one of claims 1 to 3, characterised in that the pH in step (a) is maintained for a period of 5 minutes to 30 minutes.

5. Process according to one of claims 1 to 4, characterised in that the pH in step (a) is carried out at a temperature of 20 to 30°C.

6. Process according to one of claims 1 to 5, characterised in that the pH in step (b) is in the range from 7.4 to 8.5.

7. Process according to one of claims 1 to 6, characterised in that the pH in step (b) is maintained for a period of 5 minutes to 60 minutes.

8. Process according to one of claims 1 to 7, characterised in that the pH in step (b) is carried out at a temperature of 20 to 30°C.

9. Process according to one of claims 1 to 8, characterised in that the liquid is liquid from a mammalian cell culture.

10. Process according to claim 9, characterised in that the liquid is cell-free liquid from a mammalian cell culture.