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# Method for recovering lactoperoxidase from milk

## FIELD OF THE INVENTION

The invention is in the field of the food industry and relates to a method for the recovery of lactoperoxidase from raw milk.

## PRIOR ART

The enzyme lactoperoxidase (LPO) occurs in most animals and is excreted in human beings from the mammary glands, salivary glands and mucous glands of the bronchi. It catalyses the oxidation of phenols and different anions by hydrogen peroxide. The reaction products are highly reactive molecules, which are toxic to microorganisms entering the body. LPO is therefore a part of the innate (non-specific) immune system and makes possible the neutralisation of bacteria in the milk and other mucosal secretions. The structure of the lactoperoxidase consists primarily of α-helices. Additionally, there are two short antiparallel β-sheets. A haem cofactor is located close to the centre of the apoprotein. The lactoperoxidase catalyses the oxidation of different oxygen acceptors by hydrogen peroxide:

(1) reduced acceptor + $H_2O_2$ → oxidised acceptor + $H_2O$

Examples of such oxidation reactions are:

(2) thiocyanate ($SCN^-$) → hypothiocyanite ($OSCN^-$)

(3) bromide ($Br^-$) → hypobromite ($BrO^-$)

(4) iodide ($I^-$) → hypoiodite ($IO^-$)

The source of the hydrogen peroxide is in many cases the reaction of glucose with oxygen in the presence of the enzyme glucose-oxidase (EC 1.1.3.4), which also occurs in saliva. The glucose in turn can arise from starch in the presence of the saliva enzyme amyloglucosidase (γ-amylase (EC 3.2.1.3)). Such oxidation products are highly reactive and strongly effective anti-bacterially. The lactoperoxidase system is capable, a whole range of aerobic and anaerobic bacteria, including microaerophilic helicobacter pylori. The effect of the lactoperoxidase system depends on specific experimental conditions. If bacteria are to be cultured under aerobic conditions on nutrient agar, once they have been exposed to the lactoperoxidase system, they do not grow. However, they grow very well on blood agar under anaerobic conditions. The lactoperoxidase system seems to act synergistically with lactoferrin and lysozyme. If hydrogen peroxide is present in excess of the thiocyanate, the lactoperoxidase system can also act cytotoxically. Due to the antibacterial

effectiveness of the lactoperoxidase system it is used for preserving foodstuffs and cosmetics as well as in ophthalmology. Further applications can be found in the areas of dentistry and treatment of wounds. The lactoperoxidase system can possibly also be used combatting tumours and viruses. The lactoperoxidase system inhibits the growth of bacterial flora in milk and in milk products. The addition of hydrogen peroxide and a thiocyanate extends the shelf life of cooled raw milk. The lactoperoxidase system is comparatively heat-resistant and is used as an indicator of an over-pasteurisation of milk. The lactoperoxidase system also seems to be suitable for the treatment of cavities, gingivitis and periodontitis and is therefore used as a component of tooth pastes and mouth rinsing solutions. Because it inhibits the growth of bacteria in the oral cavity, it also inhibits the acid production of said bacteria.

Lactoperoxidase is therefore a very interesting natural active substance, which can be recovered up to now only with great effort in sufficient purity from special milk fractions with the aid of chromatographic methods. Thus, for example, **EP 1401289 B1** (UPFRONT) describes a method for recovering lactoperoxidase from skimmed milk, but also sweet whey and other milk components, by using EBA (Expanded Bed Adsorption), in which 54 mg product of unknown purity are obtained per litre of feedstock. In respect to the commercial value of the product, on the one hand, and the low concentrations, in which, on the other hand, it is contained in the milk, a high interest exists in improving the yields.

A purification of lactoperoxidase with the aid of hydrophobic interaction chromatography is described in **CN 103709246 A** (BEIJING BIOTECHNOLOGY), but is only to be realised for the laboratory scale, since a production would be too expensive.

The problem addressed by the invention has thus consisted of developing a method suitable for commercial production that takes into account the above-described requirements and makes available lactoperoxidase from milk in comparatively higher yields and higher purity with the least possible technical effort.

## DESCRIPTION OF THE INVENTION

Subject matter of the invention is a method for recovering lactoperoxidase, in which

(a) raw milk is subjected to a cold separation at a temperature of from about 8 to about 15 °C and the cream is separated,

(b) the cold separated skimmed milk is subjected to Expanded Bed Adsorption (EBA), wherein the lactoperoxidase is adsorbed in the EBA column, and then

(c) the lactoperoxidase is re-eluted, desalted, dehydrated and isolated as a dry product.

It was surprisingly found that the combination of cold separation and EBA constitutes a suitable method in order to recover lactoperoxidase with a low technical effort in high yields and high purity from raw milk with the highest content of lactoperoxidase within the group of the milk fractions. Through suitable selection of the eluent in the EBA yields and purity can be further increased.

**COLD SEPARATION**

In the method according to the present invention it is provided that the temperature of the cold state of the raw milk is set by heat exchange with a heat transfer medium to an optimal value for its separation. Normally, the raw milk is made available in a cooled state, the temperature of which does not correspond to the value, in which its cold separation can be carried out most effectively and most gently for the milk fat (cream). Therefore, it is set by the heat exchange to the optimal value for its separation. The exchange cooling resulting thereby can be made available in particular by a so-called heat see-saw of other processes, which occur within a dairy. For example, the temperature of the cooled raw milk is not higher than 6 °C, while the optimal temperature for the cold separation is in the range of 8 to 15 °C and in particular 8 to 12 °C. In this event the heat exchange takes place by heating the raw milk so that the temperature of its cold state is elevated to a value lying in this range. Normally, in dairies there is a surplus of heat. Therefore, low temperature water, which arises in processes of the dairy, can be used as a heat transfer medium for the heating. This low temperature water is fed to the heat exchange process with a temperature, which for example, is in the range of 35 °C, and is cooled by the heat exchange to a temperature, which, for example, is in the range of 11 to 15 °C. Thus, the method according to the present invention makes available an important cold source for processes of the dairy.

The separation of solids as well as the skimming off of the fat content of approximately 4 wt. % usually take place in a downstream component, preferably a separator. Such components are sufficiently known from the prior art. In the milk industry separators of the company GEA Westfalia Separator GmbH are quite common, with which the separation of solids can be carried out individually or jointly (http://www.westfalia-separator.com/de/anwendungen/molkereitechnik/milch-molke.html). Preferred cold milk separators are offered by this manufacturer under the designation “Procool”. Corresponding components are also described, for example, in **DE 10036085 C1** and **DE 10361526 B3** (Westfalia) and are best known to a person skilled in the art, so that the implementation of these method steps requires no explanations, since they can be attributed to the general knowledge of the art.

EXPANDED BED ADSORPTION

The principles, according to which proteins are bound in accordance with Expanded Bed Adsorption (EBA), are not different in principle from those of a classical column chromatography or ion exchanger technology. Where the classical column chromatography, however, uses a solid phase in the form of a pack, the EBA uses the particles in a fluidised state, that is, in a kind of fluidised bed, wherein the volume is expanded ideally by a factor of 2. Furthermore, it is essential that the particles have different sizes and densities, whereby a particle size gradient arises during the expansion of the bed. In the fluidised bed additional local turbulences thereby arise. This leads in particular to components, which exceed a specific size or have the tendency to agglomerate, flowing through the fluidised bed, which would not be possible in a solid pack.

During the EBA the columns, which are preferably solid adsorption columns, are filled from above with the adsorbent. These are, on the one hand, a non-permeable material of high density and, on the other hand, an ion exchange resin capable of protein binding. Preferably, the non-permeable material has a density of at least 1.5 g/ml and/or a particle size of at most 150 µm and in particular 40 to 120 µm. Common examples of this are glass- or metal beads, especially tungsten, wherein these should have different diameters and densities as described at the outset, so that a corresponding gradient arises in the fluidised bed. This material constitutes the carrier in the fluidised bed.

The second part of the adsorber is an ion exchange resin, which is capable of binding proteins. For example, a sulfonic acid anion exchange resin based on polysaccharide is possible, such as it is described in EP 1401289 B1 (example 3). Preference is given to a tungsten carrier, which is coated with the resin.

In principle, carriers and ion exchangers can be filled into the column one after the other or together. It is also possible to coat the carrier with the resin. After the filling the carrier materials form a dense fill, which offers large aggregates little space to pass through. As soon as the bed is placed in a fluidised state, for example, by whirling up the fill with a magnetic stirrer, and the rate of sinking corresponds to the upward flow, the carriers form a stable concentration gradient. The objective is to set the flux, in which the speed of the substances to be separated is greater than that of the carrier particles. In order to increase the difference in the rising speeds, the carriers are also often provided with a quartz or metal core.

In order to remove impurities it has proven to be advantageous that the EBA column is equilibrated with the cold separated skimmed milk before and after the loading, for example, with water or a buffer solution. Further advantageous embodiments of this method step consist in that

- the cold-separated skimmed milk is applied at a flux rate of about 3 to about 50 cm/min; and/or
- the non-permeable material of high density and the ion exchange resin capable of protein binding are used in a weight ratio of about 20:80 to about 50:50; and/or
- the adsorbent consisting of the non-permeable high-density material and the ion exchange resin capable of protein binding is applied relative to the cold-separated skimmed milk to be loaded onto the EBA column in a v/v ratio of about 1:100 to about 1:1000, and preferably about 1:250 to about 1:500.

After the task is completed, the fluidised state is ended and the pack is given the opportunity to settle again. Then the rinsing of the column with water takes place and the actual desorption of the lactoperoxidase preferably in a counter-current direction, that is, from below to above, wherein preferably a dilute base is used as eluent. It has proven to be particularly advantageous with respect to the increase of the yield, if diluted base is used for this purpose, which contains a buffer. Diluted (e.g., 0.1 M) sodium hydroxide solution buffered with (e.g., 0.3 M) sodium chloride constitutes an excellent system.

In summary, the method according to the present invention comprises the following steps:

(a) separating the cream from raw milk at a temperature in the range of about 8 to about 15 °C;

(b) loading a solid adsorption column with an adsorbent consisting of a non-permeable high density material and an on exchange resin capable of protein binding;

(c) placing the adsorbent in the column in a fluidised bed state;

(d) optionally rinsing the column with water;

(e) applying the cold-separated skimmed milk from step (a);

(f) optionally rinsing the column with water;

(g) eluting the lactoperoxidase from the adsorbent in the column with dilute base, which contains a buffer;

(h) desalting and drying the eluate to obtain the lactoperoxidase as a dry solid.

**EXAMPLES**

**GENERAL APPROACH FOR THE EBA**

An EBA column with a diameter of 2 cm was placed on a magnetic stirrer and filled with 15 ml of demineralised water. The column was filled up to a height of about 20 cm with tungsten beads (diameter approx. 200 µm), which were coated with a sulfonic acid cation exchange resin of the company UpFront Chromatography A/S, Denmark (analogous to EP 1401289 B1, example 3). The magnetic stirrer was set in motion and the column was rinsed initially for 15 minutes with demineralised water with a flux of about 10 cm/min. Then different milk fractions (weight ratio adsorbent: milk fraction about 1:200) were fed and a flux of about 7.5 cm/min was set. After the entire amount of the milk fraction had passed the column, the latter was in turn rinsed for 15 minutes with demineralised water, before the lactoperoxidase was eluted with about 5 L of a diluted sodium hydroxide solution, at a flux of about 7.5 cm/min. The eluate was desalted and freed from water on a rotary evaporator. The lactoperoxidase was then obtained as a white dry powder.

**EXAMPLE 1**

1,000 ml raw milk were passed through a separator at 8 °C, wherein solid components were removed and the cream separated. The thus obtained skimmed milk had a natural pH value of 6.7 and was fed to the EBA column without further treatment. 62 mg lactoperoxidase were obtained in a purity of 93%.

**EXAMPLE 2**

Example 1 was repeated, however, a 0.1 M sodium hydroxide solution was used as eluent, which contains 0.3 M sodium chloride as buffer. 69 mg lactoperoxidase were obtained in a purity of 95%.

**COMPARISON EXAMPLE V1**

1,000 ml raw milk were fed without further separation step to the EBA column. The column material bonded due to the high fat content within a short period of time, so that an elution was practically no longer possible.

**COMPARISON EXAMPLE V2**

1,000 ml raw milk passed at about 50 °C through a combination of plate heat exchanger and separator, wherein solid components were removed and the cream was separated. The thus obtained skimmed milk had a natural pH value of 6.7 and was then pasteurised at 75 °C over a

time period of 30 seconds and then fed to the EBA column. Only 49 mg lactoperoxidase were obtained in a purity of 92%.

**COMPARISON EXAMPLE V3**

Comparison example V2 was repeated, however, a 0.1 M sodium hydroxide solution was used as eluent, which contains 0.3 M sodium chloride as buffer. 54 mg lactoperoxidase were obtained in a purity of 93%.

The examples and comparison examples show that raw milk, which has the highest content of lactoperoxidase, is unsuitable for use in chromatographic methods due to high fat content. Skimmed milk, which is produced according to the conventional method of heat separation, can be used in chromatography, however, due to the heat instability of the lactoperoxidase provides significantly lower yields than skimmed milk from the cold separation. Finally, through the specific selection of the eluent, the yield of the lactoperoxidase can be significantly influenced.

**Patentkrav**

**1.** Fremgangsmåde til at udvinde lactoperoxidase omfattende:

(a) at underkaste råmælk for en koldseparation ved en temperature fra 8 til 15 °C og at separere fløden;

(b) at underkaste den koldseparerede skummetmælk for Expanded Bed Adsorption (EBA), hvor lactoperoxidasen adsorberes af adsorptionsmidlet i EBA-søjlen; og derefter

(c) re-elueres, afsaltes, dehydreres og isoleres lactoperoxidasen som et tørprodukt.

**2.** Fremgangsmåde ifølge krav 1, **kendetegnet ved, at** råmælken skummes af ved en temperatur på fra 8 til 12 °C.

**3.** Fremgangsmåde ifølge kravene 1 og/eller 2, **kendetegnet ved, at** der anvendes en adsorptionssøjle med fast leje.

**4.** Fremgangsmåde ifølge mindst et af kravene 1 til 3, **kendetegnet ved, at** EBA'en udføres med et adsorptionsmiddel bestående af et ikke-permeabelt materiale med høj densitet og en ionbytningsresin, der er i stand til proteinbinding.

**5.** Fremgangsmåde ifølge krav 4, **kendetegnet ved, at** det ikke-permeable materiale har en densitet på mindst 1,5 g/ml og/eller en partikelstørrelse på højest 150 µm.

**6.** Fremgangsmåde ifølge krav 5, **kendetegnet ved, at** det ikke-permeable materiale er glas- eller metalbeads.

**7.** Fremgangsmåde ifølge krav 4, **kendetegnet ved, at** ionbytningsresinen er en sulfonsyre-anionbytningsresin.

**8.** Fremgangsmåde ifølge mindst et af kravene 1 til 7, **kendetegnet ved, at** EBA-søjlen skylles før og efter påfyldningen den koldseparerede skummetmælk.

**9.** Fremgangsmåde ifølge mindst et af kravene 1 til 7, **kendetegnet ved, at** den koldseparerede skummetmælk tilføres ved en fluxhastighed på omkring 3 til omkring 50 cm/min.

**10.** Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 9, **kendetegnet ved, at** det ikke-permeable materiale med høj densitet og ionbytningsresinen, som er i stand til proteinbinding, anvendes i et vægtforhold på omkring 20:80 til omkring 50:50.

**11.** Fremgangsmåde ifølge mindst et af kravene 1 til 10, **kendetegnet ved, at** adsorptionsmidlet bestående af det ikke-permeable materiale med høj densitet og ionbytningsresinen, der er i stand til proteinbinding, påfyldes i forhold til den koldseparerede skummetmælk, der skal fyldes på EBA-søjlen, i et v/v-forhold på omkring 1: 100 til omkring 1: 1000.

**12.** Fremgangsmåde ifølge mindst et af kravene 1 til 10, **kendetegnet ved, at** lactoperoxidasen elueres med fortyndet base.

**13.** Fremgangsmåde ifølge krav 12, **kendetegnet ved, at** der anvendes en fortyndet base, som indeholder en buffer.

**14.** Fremgangsmåde ifølge kravene 12 og/eller 13, **kendetegnet ved, at** der anvendes fortyndet natriumhydroxidopløsning bufret med natriumchlorid.

**15.** Fremgangsmåde ifølge krav 1 omfattende trinnene:

(a) at separere fløden fra råmælk ved en temperature i området fra omkring 8 til omkring 15 °C;

(b) at fylde en fast adsorptionssøjle med et adsorptionsmiddel bestående af det ikke-permeable materiale med høj densitet og en ionbytningsresin, der er i stand til proteinbinding;

(c) at anbringe adsorptionsmidlet i søjlen i en tilstand med fluidiseret leje;

(d) eventuelt af skylle søjlen med vand;

(e) at tilføre den koldseparerede skummetmælk fra trin (a);

(f) eventuelt at skylle søjlen med vand;

(g) at eluere lactoperoxidasen fra adsorptionsmidlet i søjlen med fortyndet base, der indeholder en buffer;

(h) at afsalte og tørre eluatet for at opnå lactoperoxidasen som et tørt faststof.