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Lysosubtilin modification, Fermosorb, designed for polymeric carrier-mediated intestinal delivery of lytic enzymes: pilot-scale preparation and evaluation of this veterinary medicinal product

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Abstract

Antimicrobial enzymotherapy/enzymoprophylaxis has potential for use as a measure to overcome problems associated with resistance to commonly applied antibiotics. Lysosubtilin, an authorized veterinary medicinal product, when used per os for the treatment and prophylaxis of intestinal infections in newborn calves, is not always efficient due to partial inactivation of lytic enzymes in the gastric region. In this contribution a simple technology for preparation of pH-dependent reversibly dissociating acid stable enzyme-polymer complex (two-component oral delayed-release lysosubtilin formulation, Fermosorb) designed for intestinal delivery of lytic enzymes is described. The technology is based on immobilization of lytic enzymes, using 1% lysosubtilin solution in 10 mM acetate buffer of pH 5.0, onto commercial highly porous carboxylic cation exchanger Biocarb L (v/w ratio 10:1, process duration 1 h) with after-following procedures of vacuum-filtration, oven-drying and standardization of the enzyme-polymer complex formed. The technology process of pilot-scale Fermosorb fabrication on the whole revealed itself as simply employed and highly repeatable, totalling in the final lytic enzyme activity yield of 40.2% (the average value obtained from the analysis of the 11 batches running) and $\sim$4000 (3938) kg of Fermosorb (200 batches) produced. The proposed technological approach can be successfully applied for fabrication of other enzyme preparations as well and this was shown in the example of Polyferm, a preparation with both lytic and proteolytic enzyme activities. In vitro evaluation of Fermosorb revealed it was more stable when exposed to the acidic environment as well as in storage when compared with the native lysosubtilin. No negative change in the antimicrobial spectrum of action of Fermosorb versus lysosubtilin, influenced by immobilization of lytic enzymes onto Biocarb L, was observed. Moreover, all six lysosubtilin-resistant microbial strains tested have been found to be Fermosorb-susceptible. In vivo evaluation studies performed on 1200 newborn calves revealed 95.2% therapeutic as well as 95.0% prophylactic efficacy of Fermosorb in respect to colibacillosis versus 74.0 and 80.0% for lysosubtilin, respectively, the differences being statistically significant ($P < 0.01$). As a consequence of these studies Fermosorb was authorized for use throughout the former Soviet Union. Data collected during postmarketing surveillance of Fermosorb, which was applied for more than 163 000 newborn calves, confirmed high efficacy (92.3 and 95.5% for treatment and prophylaxis, respectively) and safety of this veterinary medicinal product.

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1. Introduction

In recent years the emergent resistance to antimicrobial drugs has become the topic of worldwide discussion (Neu, 1992; Kunin, 1993; Verhoef, 1994; Chadwick and Goode, 1997). Different strategies have been proposed to overcome the situation, the appropriate use of known antimicrobial agents and the creation of novel, replacing ineffective, agents being among the main issues (Guglielmo, 1995; Keck and Borne, 1995; Araneo et al., 1996; Moellering, 1998).

Lytic enzyme preparations are antimicrobial agents directly degrading the cell walls of microorganisms and thus differing by their mode of action (Ghuysen et al., 1966; Strominger and Ghuysen, 1967; Fuglsang et al., 1995) from the commonly used antibiotics. Some representatives, such as lysozyme, lysostaphin, lysosubtilin, lysoamidase, and mutanolysin have already been shown to be highly perspective for human and/or animal use (Biziulevichius and Kislukhina, 1988; Blackburn and Pollack, 1988; Severin and Tomasz, 1995; Sava, 1996; Climo et al., 1998).

The technology background for manufacturing of lysosubtilin, an acetone precipitated preparation of lytic enzymes from *Bacillus subtilis*, was described in 1976 (Biziulevichius et al., 1976). Since 1983 it has been produced on an industrial scale especially for veterinary purposes. Lysosubtilin is a complex enzyme preparation composed of two lytic endopeptidases with rather distinct features (Biziulevichius et al., 1995), which acting jointly on proteinaceous components of microbial cell envelope are responsible for very broad antimicrobial spectrum action of lysosubtilin. Information on the physicochemical properties, and antibacterial and antifungal spectrum of action of lysosubtilin has already been published (Biziulevichius et al., 1987, 1989, 1995). Its efficacy for the prophylaxis and treatment of gastrointestinal disorders in newborn calves as well as for the treatment of gynaecological diseases in cows has been shown (Biziulevichius and Arestov, 1997a; Biziulevichius and Lukauskas, 1998a,b). Its safety when used by oral route of administration has also been demonstrated (Biziulevichius and Arestov, 1997b). Since 1990, lysosubtilin has been listed among the preparations approved for use in veterinary medicine throughout the former Soviet Union and about 1.5 million animals have already been cured (or protected) by applying this preparation (Biziulevichius and Lukauskas, 1998b).

Lytic enzymes are labile chemical compounds (the feature that is characteristic for many proteins) and their clinical use has several peculiarities when compared with the usual antimicrobial drugs. Their oral application requires that specific measures aimed at preserving enzyme activities be implemented. Lysosubtilin is most active at pH 7.2 (Biziulevichius et al., 1987): thus, to avoid the inactivation of lytic enzymes in the gastric region when given per os for prophylaxis and treatment of gastrointestinal disorders in newborn calves, it was recommended to be used together with colostrum or milk, both supplemented with 10 g/l of sodium bicarbonate (Biziulevichius and Arestov, 1997a). In general, this measure justified itself, though the postmarketing surveillance data analysis revealed that in case of typical intestinal infections, such as colibacillosis, it was not always enough. At this stage the necessity for design of an acid resistant delayed-release lysosubtilin formulation for the intestinal delivery of lytic enzymes has pointed it out.

Oral acid resistant enzyme preparations are conventionally prepared in the form of tablets or pellets coated with enteric polymers (Mayer and Viernstein, 1994; Schulz and Schmidt, 1995; Kleine, 1997). Theoretically, these can also be prepared by immobilization techniques employing the same type polymers (showing reversibly soluble-insoluble characteristics with pH change) or water insoluble polymers capable of forming en-

zyme-polymer complexes stable in acidic environment and reversibly dissociating at neutral pH values. If one foresees the future development of an oral delayed-release lytic enzyme preparation in this way (as far as known there are no such preparations, except as described in this study) some technology achievements on immobilization of antimicrobial enzymes lysozyme and chitinase (Crapisi et al., 1993; Chen and Chang, 1994; Chen and Chen, 1997) should be considered. On the other hand, preparations for veterinary needs should be designed using inexpensive commercially available materials identified as safe and manufactured using practical techniques.

Highly porous water insoluble carboxylic cation exchanger Biocarb L (henceforth referred to as Biocarb L only) was proposed for enzyme immobilization in 1986 (Samsonov et al., 1986). This is a beaded cross-linked copolymer of methacrylic acid and triethylene glycol dimethacrylate with an exchange capacity of 2.8 meq/ml or 9.8 meq/g produced on a large scale and approved for use as an atoxic carrier in the pharmaceutical industry (Laskorin, 1989). Properties of Biocarb-type ion exchangers have recently been reviewed (Samsonov and Kuznetsova, 1992).

In this contribution we describe a simple technology for the preparation of pH-dependent reversibly dissociating acid stable enzyme-polymer complex (two-component oral delayed-release lysosubtilin formulation, Fermosorb) by immobilization of lytic enzymes onto Biocarb L. The description is presented in a step-by-step manner with particulars of the immobilization procedure (pH, process duration) and observations on lytic enzyme activity changes in the course of the pilot-scale preparation of Fermosorb being emphasized. The in vitro and in vivo evaluation results in newborn calves of this veterinary medicinal product including data collected during postmarketing surveillance are also given.

2. Materials and methods

2.1. Materials

Lysosubtilin (more precisely Lysosubtilin G10 ×) is a commercial preparation of lytic enzymes from *B. subtilis* SK-52 and was acquired from the State Joint-Stock Enterprise 'Biosinteze' formerly called the Vilnius Experimental-Industrial Plant of Enzyme Preparations (Vilnius, Lithuania). This product was a powder of light (greyish or yellowish) colour with a lytic activity 10^6 U/g. For unit (U) definition see Section 2.2.6.

Biocarb L was purchased from the All-Union Research Institute of Chemical Technology (Moscow, Russia). According to our instructions the manufacturer prepared the polymeric beads with mechanically destroyed outer surface to make the penetration of enzymes into pores easier and the beads were properly washed several times, with a final pH of washing medium being near 5.0. The polymer (colour: white; wet bead size: 0.5–2.0 mm; moisture content: ~60%) was obtained in sealed plastic sacks, each containing at least 25 kg of the product.

All other reagents were of reagent grade. Distilled water was always used.

2.2. Fabrication of Fermosorb

In order not to forestall our own study results, some values in this section are not finalized but defined as 'optimal'. In such cases a reference to Section 3.1 (Results and discussion) is always given.

2.2.1. Preparation of lysosubtilin solution

The powder of lysosubtilin was dissolved in 10 mM acetate buffer of different pH values in initial studies and then later of an optimal for immobilization pH one (Section 3.1) to form a 1% (w/v) solution.

2.2.2. Preparation of Biocarb L

The same buffer solutions as described in Section 2.2.1 were used to equilibrate Biocarb L. In any laboratory experiment or pilot-scale procedure the buffer of pH value strictly corresponding to that employed to prepare the lysosubtilin solution was applied.

2.2.3. Determination of optimal pH value and process duration for immobilization of lytic enzymes onto Biocarb L

A total of six 5-g aliquots of Biocarb L each, equilibrated with the 10-mM acetate buffer of pH values 4.4, 4.6, 4.8, 5.0, 5.2 and 5.4, respectively, were added to 50-ml aliquots of lysosubtilin solution of the corresponding pH value. An immobilization procedure was carried out for 1 h at room temperature under constant agitation using a magnetic stirrer. Then uptaken lytic enzyme activity was measured.

The experiment was repeated using the buffer of the estimated optimal pH value (Section 3.1) only (one reaction mixture), while the duration of the immobilization procedure was prolonged up to 5 h. The uptaken lytic enzyme activity was measured every half an hour.

2.2.4. Determination of optimal pH value and process duration for desorption of lytic enzymes from Biocarb L

A total of six 5-g aliquots of the enzyme-polymer complex each, prepared as described in Section 2.2.3 under optimal conditions of immobilization (Section 3.1), were dispersed in 50-ml aliquots of 50-mM phosphate buffer of pH values 6.6, 6.8, 7.0, 7.2, 7.4 and 7.6, respectively, and desorption procedure was carried for 1 h at room temperature under constant agitation using a magnetic stirrer. Then released lytic enzyme activity was measured.

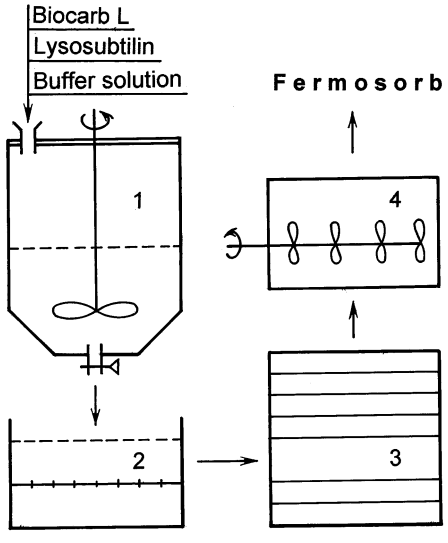

Fig. 1. Scheme representing the principal technological points of the pilot-scale preparation of Fermosorb. (P.1) Reaction vessel; (P.2) vacuum filter; (P.3) oven; (P.4) blender. Other operations (polymer matrix preparation, quality control, etc.) are not illustrated.

The experiment was repeated using the buffer of the estimated optimal pH value (Section 3.1) only (one reaction mixture), while the duration of the desorption procedure was prolonged up to 2 h. The released lytic enzyme activity was measured every 15 min.

2.2.5. Pilot-scale preparation of Fermosorb

A total of 250 l of the lysosubtilin solution (Section 2.2.1) was prepared under constant mechanical agitation with a propeller stirrer (not less than 600 rpm) in a 0.6-m^3 closed metal vessel (Fig. 1, P.1) having an inert plastic-coated inner surface and two holes (one in a cap for pouring the reaction components in and another one, equipped with a bolt, in the bottom for pouring the mixture out at the end of an immobilization process). The dissolution stage lasted for about half an hour. Then 25–30 kg of Biocarb L ready for the immobilization procedure (Section 2.2.2) were added and the process was performed under constant agitation at room temperature and at optimal conditions for immobilization (Section 3.1). The reaction mixture was vacuum-filtered through a filter cloth membrane previously set on a stainless filter holder (Fig. 1, P.2). The filtered beads were dispersed on shelves and dried in a well-ventilated oven (Fig. 1, P.3) at 45–50°C overnight. Then lytic activity of the dried enzyme-polymer complex was measured and for the consumer's convenience it was mixed in a blender (Fig. 1, P.4) with a certain amount of an oven-dried Biocarb L to form 5×10^4 U/g final activity of Fermosorb.

2.2.6. Measurement of lytic enzyme activity

Lytic activity was measured according to Kislukhina (1976)). A unit (U) is defined as follows: 1 U generates a decrease in turbidity of 0.001 OD min^{-1} at 520–540 nm in a suspension of *Escherichia coli* K12 dried cells (initial OD 0.6)

in 1.0 ml of 4 mM phosphate buffer, pH 7.2 at 30°C.

Activity of enzyme-polymer complexes was measured after 1 or 2 h desorption of lytic enzymes with an approximately tenfold amount of 50 mM phosphate buffer, pH 7.2, for wet and dry samples, respectively.

2.3. Evaluation of Fermosorb

2.3.1. In vitro studies

Samples of the enzyme preparations Fermosorb and lysosubtilin prior to determination of their stability in acidic environment were prepared as follows. Fermosorb was allowed to swell for 1 h in a tenfold amount of 10 mM acetate buffer of pH 5.0, while a sample of lysosubtilin used was a freshly prepared 1% solution in the same buffer. The samples were each divided equally into six parts, which were adjusted, where appropriate, with 10% hydrochloric acid to pH values of 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0, respectively, and kept at 37°C for 1 h under constant agitation using a magnetic stirrer. Then residual lytic enzyme activity of the lysosubtilin solution and Fermosorb was measured.

For determination of stability in storage 20 identical samples each of Fermosorb and lysosubtilin were prepared in hermetically sealed plastic bags. Lytic activity was measured at preparation of the samples (zero time) and later at 1-year intervals with the new bags being opened every test time.

Preparation of microbial cells used as substrates and determination of antimicrobial spectrum of Fermosorb action were the same as described previously for lysosubtilin (Biziulevichius et al., 1989, 1995). The extent of lysis of substrates was evaluated according to the decrease of optical density of their suspensions after incubation with lytic enzymes for 1 h at 37°C. To make the results comparable with those achieved for lysosubtilin solution with the activity of 1000 U/ml (Biziulevichius et al., 1989, 1995) the initial optical density of the reaction mixture in 4 mM phosphate buffer of pH 7.2 was ~ 0.6, while activity of samples obtained by desorption of lytic enzymes from Fermosorb was also 1000 U/ml. In control samples the enzyme was absent. The results were expressed as the difference in the percentage of decrease of the optical density in the test and control samples, which corresponded to the percentage of lysis of the substrate by the enzyme preparation excluding the percentage of autolysis.

2.3.2. In vivo studies

Experiments with newborn calves were approved by the former Soviet Union (FSU) Chief Veterinary Medicine Board of Ministry of Agriculture. The trials were performed in the farms of three FSU republics (Byelarus, Lithuania, Russia) on 1200 animals. The calves were maintained under usual industrial breeding conditions. The prevalence of colibacillosis among calves of the different farms at the disease outbreaks (the time when the experiments were performed) varied considerably, but it was not lower than 50%. The calves were randomly divided into groups. Clinical observation of the calves was performed at least every 8 h throughout the experiments.

The calves in the treatment groups were those in which the first signs of colibacillosis (Fraser et al., 1991) had been detected (generally on the 2nd or 3rd day of life). The study group calves were treated with Fermosorb in the authorized manner proposed by us previously (Zotkin et al., 1987a), i.e. they were given the drug in the dose 10^4 U/kg of weight immediately after calf initiation into the study and then later in the same dose three times daily (every 8 h) until recovery, for no longer than 3 days. Fermosorb was administered being dispersed in 50 ml of boiled water or weak tea using a nipple bottle. The control group calves were treated with lysosubtilin by the previously described authorized method (Biziulevichius and Arestov, 1997a), i.e. they were given the drug in the dose 2.5×10^4 U/kg of weight in milk supplemented with 10 g/l of sodium bicarbonate twice daily, for no longer than 3 days. After the 3-day period unsuccessfully treated calves of both groups were withdrawn from the experiment (unless the calves died) and were further treated in other ways or culled.

The calves of the prophylaxis groups were healthy newborn ones. The prophylaxis of the

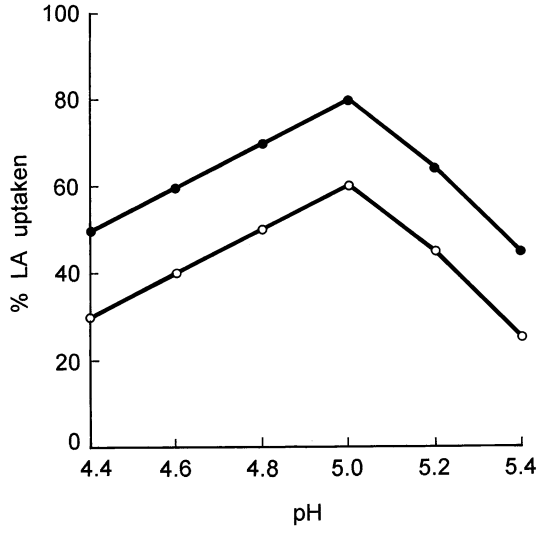

Fig. 2. Effect of pH on the percentage of lytic enzyme activity (LA) uptaken, after 1 h, from reaction mixture by Biocarb L. Experiment was carried out in 10 mM acetate buffer. The LA uptake was evaluated either by analysis of a liquid phase, i.e. solution containing lytic enzymes (solid circles, here and in other figures) or by the analysis of a solid phase, i.e. enzyme-polymer complex formed (open circles). For taking a sample of the liquid phase with a pipette the magnetic stirrer was stopped for 1 min and a solid phase was allowed to settle, while for the analysis of the solid phase, the two phases were separated using a glass-filter.

study group calves was performed using Fermosorb as proposed by us previously (Sisyagin et al., 1988a), i.e. they were administered the drug in the same dose and by the same route as described above for the study treatment group calves with the difference that the initial dose of Fermosorb was given 90 min after the first colostrum feeding and then later twice daily (every 12 h) for 5 days. The control group calves were protected as described previously (Biziulevichius and Arestov, 1997a), i.e. they were administered lysosubtilin in the dose 2×10^4 U/kg of weight in colostrum or later milk, both supplemented with 10 g/l of sodium bicarbonate twice a day, starting with the first feeding (30–45 min after birth), for 5 days. Unsuccessfully protected calves of both groups, as soon as the first signs of colibacillosis (Fraser et al., 1991) appeared, were withdrawn from the experiment and treated.

3. Results and discussion

3.1. Fabrication of Fermosorb

Baseline studies revealed that our choice of the 1% lysosubtilin solution, further used in the pilot-scale preparation of Fermosorb (an abbreviation of Ferment + sorbent), was correct for at least two reasons. First, such a solution is more stable when compared those of lower or higher concentration (theoretical speculations were confirmed by examining stability of 0.5, 1, 2, 3 and 5% lysosubtilin solutions in storage at room temperature). Second, lytic enzyme activity of the solution (10^4 U/ml) is near that (1.3×10^4 U/ml) obtained by submerged cultivation of the industrial lysosubtilin producer (Biziulevichius and Normantiene, 1997), making the future substitution of the lysosubtilin solution by culture liquid hopeful from the economical point of view. The advantage of a 1:10 ratio (w/v, approximate) of Biocarb L and the lysosubtilin solution for fabrication of Fermosorb was determined experimentally once more considering economy (further enlargement of the enzyme' s part in the reaction mixture was not cost-effective).

Optimal pH value for immobilization of lytic enzymes onto Biocarb L was found to be 5.0 (Fig. 2). At any pH value tested the percentage lytic enzyme activity uptaken was about 20% higher when analysed according to the difference of the initial activity of the lysosubtilin solution and the activity of the supernatant after the immobilization process in comparison with results obtained by the direct analysis of enzyme-polymer complex formed (separate curves in Fig. 2). The 20% difference of the percentage lytic enzyme activity uptaken may be attributed either to inactivation of lytic enzymes by polymeric structures during immobilization (absorption or adsorption) or to formation of a complex with irreversibly bound enzymes. Either way, the percentage immobilization at pH 5.0 regarding lytic enzyme activity remains not less 60% (lower curve in Fig. 2). We will return to the latter value shortly when discussing losses of lytic enzyme activity in the course of the pilot-scale preparation of Fermosorb.

When the percentage lytic enzyme activity uptaken was investigated in relationship with duration of the immobilization process, the linear increase was observed until 1 h (Fig. 3) and the

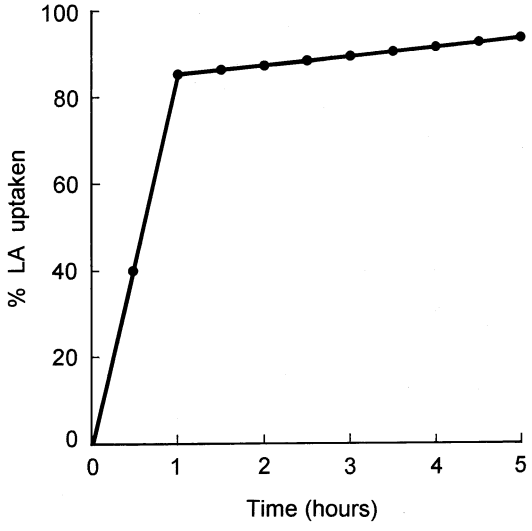

Fig. 3. Effect of time on the percentage of lytic enzyme activity (LA) uptaken from reaction mixture by Biocarb. Experiment was carried out in 10 mM acetate buffer of pH 5.0.

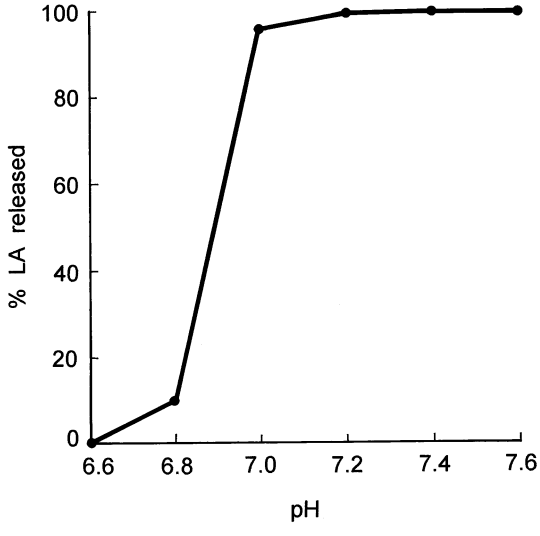

Fig. 4. Effect of pH on the percentage of lytic enzyme activity (LA) released, after 1 h, from the enzyme-polymer complex. Experiment was carried out in 50 mM phosphate buffer. Here and in Fig. 5 the highest value of the percentage LA released was considered to be 100%.

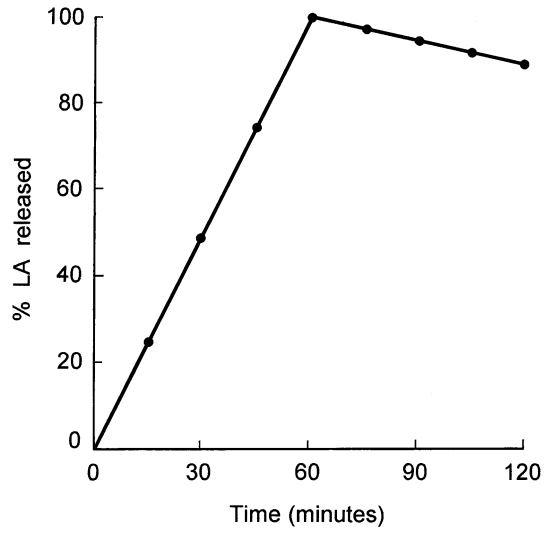

Fig. 5. Effect of time on the percentage of lytic enzyme activity (LA) released from the enzyme-polymer complex. Experiment was carried out in 50 mM phosphate buffer, pH 7.2.

1-h duration was determined as technologically optimal. Moreover, further insignificant increase of the percentage lytic enzyme activity uptaken may be considered as being an artifact associated with a decrease of remaining activity of lysosubtilin solution as a function of pH (the stability of the lysosubtilin solutions of different pH values including pH 5.0 in comparison with Fermosorb will be discussed in Section 3.2.1 as well as shown in Table 2).

The desorption profile of lytic enzymes from the enzyme-polymer complex is given in Figs. 4 and 5. The percentage lytic enzyme activity released in 50 mM phosphate buffer grew rapidly at pH values approaching neutral and reached its maximum at pH 7.2 (Fig. 4) after 1 h (Fig. 5). Further observed decrease of the percentage lytic enzyme activity released with respect to desorption process duration is, most probably, associated with the influence of the ionic strength of the buffer on the lytic enzyme activity (Biziulevichius et al., 1987). That is why the 1-h duration desorption process applying 50 mM phosphate buffer of pH 7.2 is being used in the control of lytic enzyme activity of the enzyme-polymer complex. Here it may be of some interest to add that, when other hydrolytic enzymes were applied to form enzyme-

polymer complexes with Biocarb L, the optimal enzyme desorption parameters were the same, though their immobilization had to be performed differently, e.g. when using culture liquid of *B. subtilis* as enzyme source, sorption of amylolytic enzymes had to be performed for 2 h at pH 4.4, while optimal conditions for sorption of proteolytic enzymes were pH 4.6 and 8-h duration (Biziulevičius and Žukaitė, unpublished data).

After determination of the optimal conditions of immobilization (as well as desorption) and fulfilment of scaling-up procedures pilot-scale fabrication of Fermosorb was organized. Fabrication was performed as described in Section 2.2.5. Changes of lytic enzyme activity in the course of the pilot-scale preparation of Fermosorb are given in Table 1. The main losses of lytic enzyme activity were observed at immobilization stage (40%) and at drying (20%), totalling in the final lytic enzyme activity yield of near 40%. Our attempts to increase the activity yield by variation of drying conditions (freeze-drying, oven-drying at lower than 45°C temperatures, etc.) were unsuccessful. The technology process of Fermosorb fabrication on the whole revealed itself as simply employed and highly repeatable. About 4000 (3938) kg of Fermosorb (200 batches) have already been produced using the technology described and has all been used in veterinary medicine practice for the treatment or prophylaxis of colibacillosis in newborn calves (for application results see below).

The technological approach can be successfully applied for fabrication of other enzyme preparations as well. A total of five batches ($\sim$ 100 kg) of Polyferm (an abbreviation of Polymer + ferment) were produced in the same manner from the industrial culture filtrate of *B. subtilis*, currently used in the production of neutral proteinase, by immobilization of proteolytic enzymes onto Biocarb L at pH 4.6 for 8 h (Biziulevičius and Žukaitė, unpublished data). We will return to this proteolytic enzyme preparation, when discussing advantages of antimicrobial enzymotherapy, as Polyferm possesses rather high lytic activity towards microbial cells (Biziulevičius and Žukaitė, unpublished data). In general, antimicrobial action of proteolytic enzymes, including neutral proteinase of *B. subtilis*, has been known for a long time (Kislukhina and Shevchuk, 1976; Thorne et al., 1976; Dean and Ward, 1991; Grenier, 1994). Thus, Polyferm may be considered to be, to some extent, a one more reversibly dissociating enzyme-polymer complex designed for the intestinal delivery of lytic enzymes.

3.2. Evaluation of Fermosorb

3.2.1. In vitro studies

Oral preparations for intestinal drug delivery are to be designed in a manner ensuring the protection of an active substance in the acidic environment of the gastric region as well as its release at neutral pH values characteristic for the intestines. Retention of lytic enzyme activity in the acidic environment by the polymeric structures of Biocarb L is shown in Table 2. Our previous studies (Biziulevichius and Arestov, 1997a) have shown the abomasal acidity of newborn calves being for the most part of pH 3.5–4.0 (pH range from 2.8 to 4.9 depending on the time of food intake), thus the advantages of Fermo-

Table 1
Lytic enzyme activity (LA) changes in the course of the pilot-scale preparation of Fermosorb

Stage[a]	Amount (kg)	LA ($U \times 10^4$/g)	LA yield (%)
1% Lysosubtilin solution	250	1.0	100
Enzyme-polymer complex (wet)	28.0[b]	5.4[b]	60.5[b]
Enzyme-polymer complex (dry)	14.8[b]	6.8[b]	40.3[b]
Fermosorb	20.1[b]	5.0	40.2[b]

[a] The downstream process details are given in Section 2.2.5.

[b] The results are the average values obtained from the analysis of the 11 batches running (lot numbers from 80 to 90), with the difference between the corresponding values being no more than 10%.

Table 2
Residual lytic enzyme activity (LA) of preswollen Fermosorb and 1% lysosubtilin solution after incubation for 1 h in the acidic environment at 37°C

pH	Residual LA (%) of	
	Fermosorb	Lysosubtilin solution
2.5	83	0
3.0	90	0
3.5	96	5
4.0	100	21
4.5	100	58
5.0	100	80

sorb versus native lysosubtilin with respect to their stability in acidic environment are evident (Table 2). As for the active release of lytic enzyme activity from the enzyme-polymer complex at neutral pH values, this has already been shown in the previous section (Fig. 4). At this point, a definition of Fermosorb as the pH-dependent reversibly dissociating acid stable enzyme-polymer complex (two-component oral delayed-release lysosubtilin formulation) should be given.

Table 3
Residual lytic enzyme activity (LA) of dry Fermosorb and lysosubtilin preparations[a] after different periods of storage at room temperature

Storage period (year)	Residual LA (%) of	
	Fermosorb	Lysosubtilin
0	100	100
1	100	100
2	100	95
3	100	92
4	100	88
5	100	83
6	98	80
7	96	77
8	94	73
9	92	70
10	90	66
11	[b]	63
12	[b]	60
13	[b]	56
14	[b]	53
15	[b]	50

[a] Fermosorb and lysosubtilin were dried identically. See Section 2.2.5 for the drying conditions.
[b] To be determined in the future.

Validity period is one of the most important characteristics of any drug. Results presented in Table 3 show that no losses of lytic enzyme activity of Fermosorb were observed when stored at room temperature for 5 years versus 1 year for lysosubtilin. The further 2% yearly decrease of lytic enzyme activity observed for 5 subsequent years allows us to predict the half-life of activity retention, the criterion widely used in applied enzymology, is going to be much higher for Fermosorb than that already determined (15 years) for lysosubtilin (Table 3).

As the losses of lytic enzyme activity in the course of Fermosorb fabrication are considerable (Table 1), is was quite probable that the losses were due to only one out of the two lytic enzymes. If so, spectrum of antimicrobial action of Fermosorb might differ a lot from that of lysosubtilin. A comparison of in vitro activity of Fermosorb and lysosubtilin samples, standardized in respect to *E. coli* K12 cells, towards 58 microbial strains of various taxonomic groups showed that degree of lysis of micro-organisms achieved by action of lytic enzymes released from Fermosorb was higher (0.9–7.7% and 4.1–13.5% for bacteria and filamentous fungi/yeasts, respectively) versus those present in the lysosubtilin solution (Biziulevichius et al., 1989, 1995). Moreover, all five lysosubtilin-resistant bacterial strains (Biziulevichius et al., 1989) as well as one lysosubtilin-resistant yeast strain (Biziulevichius et al., 1995) tested were found to be Fermosorb-susceptible, showing an activating effect of Biocarb L polymeric structures on lytic enzyme activity. These findings are of great importance and will be used in future studies when keeping in mind that intestinal infections in newborn calves (target species of animals) as well as other animal neonates are always of polymicrobial aetiology (Fraser et al., 1991), in spite of the fact that the most typical pathogen affecting newborn calves is considered to be *E. coli* (Fraser et al., 1991).

3.2.2. In vivo studies

Our initial studies performed on a small number of animals have shown therapeutic efficacy of

Fermosorb, when used for the treatment of colibacillosis in newborn calves, to be 100% with an average clinical recovery period of 34 h versus 87.5% and 106 h as well as 75.0% and 84 h for gentamicin and tylosin, respectively (Zotkin et al., 1987a). Prophylactic efficacy of Fermosorb, when applied for 5 days, with respect to colibacillosis in newborn calves was shown to be 95.0% (Sisyagin et al., 1988a). Results were verified on the farms of three FSU republics on a large number of animals and the verification data are shown in Table 4. Therapeutic efficacy of Fermosorb was found to be 95.2% and this was significantly higher ($P < 0.01$) by 21.2% when compared with lysosubtilin. A clinical recovery period using Fermosorb lasted on average for 36 versus 60 h for lysosubtilin, while the number of lethal outcomes (in spite of all efforts to rescue the calves) was six (1.2%) and 22 (4.4%) in Fermosorb treated and lysosubtilin treated groups, respectively (data not shown). Prophylactic efficacy of Fermosorb (95.0%) was also higher (by 15.0%) when compared with lysosubtilin, the difference being statistically significant ($P < 0.01$).

As a consequence of the results achieved in the trials (Table 4), the Chief Veterinary Medicine Board of FSU Ministry of Agriculture in 1990 issued a licence for using Fermosorb as a therapeutic and prophylactic measure against colibacillosis in newborn calves on a large scale. Fermosorb found its way onto the list of preparations authorized for use in veterinary medicine throughout FSU. Data collected during postmarketing surveillance of Fermosorb confirmed, in general, the high efficacy (and safety) of this veterinary medicinal product, disclosing the following facts. First, almost all Fermosorb manufactured (3900 out of 3938 kg) was used by veterinary practitioners for treatment, while only the few remaining kilograms were used for prophylaxis of colibacillosis in newborn calves. Second, 150 000 out of 162 500 calves treated with Fermosorb were successfully cured (therapeutic efficacy 92.3%). Third, ~ 600 calves were protected from colibacillosis with the overall prophylactic efficacy of Fermosorb being 95.5%. Fourth, no adverse effects of Fermosorb application were observed and no complaints pertaining to low quality or inefficacy of the preparation were received.

Antimicrobial enzymotherapy/enzymoprophylaxis as a measure to overcome problems associated with resistance to commonly used antibiotics was discussed in Section 1. Results presented in this paper, data published by us previously on application of Polyferm (Zotkin et al., 1987b; Sisyagin et al., 1988b) and results achieved recently by other authors using enteric-coated bromelain, a preparation of proteolytic enzymes of plant origin, for protection against experimen-

Table 4
Efficacy of Fermosorb and lysosubtilin for the treatment and prophylaxis of colibacillosis in newborn calves[a]

Location of trials		Therapeutic efficacy[b] (number of cured animals/total, %) of		Prophylactic efficacy[c] (number of protected animals/total, %) of	
State	Region	Fermosorb	Lysosubtilin	Fermosorb	Lysosubtilin
Byelarus	Grodno	95/100 (95)*	73/100 (73)	19/20 (95)	16/20 (80)
Lithuania	Trakai	97/100 (97)*	75/100 (75)	20/20 (100)	17/20 (85)
Lithuania	Vilnius	94/100 (94)*	73/100 (73)	19/20 (95)	16/20 (90)
Russia	Kaliningrad	97/100 (97)*	77/100 (77)	19/20 (95)	16/20 (80)
Russia	Komi	93/100 (93)*	72/100 (72)	18/20 (90)	15/20 (75)
Total		476/500 (95.2)*	370/500 (74.0)	95/100 (95.0)*	80/100 (80.0)

[a] See Section 2.3.2 for the experiment details.
[b] Clinical recovery of calves was a criterion for the treatment success.
[c] Absence of colibacillosis symptoms in calves throughout the experiment period was a criterion for the prophylaxis success.
* Data are significantly different ($P < 0.01$) from lysosubtilin treated/protected group (exact Fisher test).

tally induced colibacillosis in rabbits (Mynott et al., 1991) as well as in piglets (Mynott et al., 1996; Chandler and Mynott, 1998), show that oral preparations designed for intestinal delivery of enzymes with antimicrobial action may be very useful for treatment and/or prophylaxis of this life-threatening disease in animals. Keeping in mind the broad antimicrobial spectrum of action of Fermosorb (as well as Polyferm), the struggle against other intestinal infections may also become easier.

4. Conclusions

In this work, a novel approach in design of oral preparations intended for intestinal delivery of enzymes with antimicrobial action using a polymeric carrier as a mediator is described. A simple technology based on immobilization of such enzymes onto highly porous carboxylic cation exchanger Biocarb L is given. In vitro and in vivo evaluation of pH-dependent reversibly dissociating acid stable enzyme-polymer complex Fermosorb is provided. And finally, advantages and future perspectives of antimicrobial enzymotherapy/enzymoprophylaxis, including application of enzyme-polymer complexes manufactured, as a measure to overcome problems associated with resistance to commonly used antibiotics, are discussed.

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References

Araneo, B.A., Cebra, J.J., Beuth, J., Fuller, R., Heidt, P.J., Midvedt, T., Nord, C.E., Nieuwenhuis, P., Manson, W.L., Pulverer, G., Rusch, V.C., Tanaka, R., Van Der Waaij, D., Walker, R.J., Wells, C.L., 1996. Problems and priorities for controlling opportunistic pathogens with new antimicrobial strategies: an overview of current literature. Zentralbl. Bakteriol. 283, 431–465.

Biziulevichius, G.A., Arestov, I.G., 1997a. In vivo studies on lysosubtilin. 1. Efficacy for prophylaxis and treatment of gastrointestinal disorders in newborn calves. Vet. Res. 28, 19–35.

Biziulevichius, G.A., Arestov, I.G., 1997b. Safety of lysosubtilin per os in mice, rabbits and calves. Vet. Res. 28, 385–395.

Biziulevichius, G.A.S., Kislukhina, O.V., 1988. Lytic enzymes in veterinary medicine. A review. Veterinariya (Moscow) 3, 56–59 in Russian.

Biziulevichius, G.A., Lukauskas, K., 1998a. In vivo studies on lysosubtilin. 2. Efficacy for treatment of post-partum endometritis in cows. Vet. Res. 29, 47–58.

Biziulevichius, G.A., Lukauskas, K., 1998b. In vivo studies on lysosubtilin. 3. Efficacy for treatment of mastitis and superficial lesions of the udder and teats in cows. Vet. Res. 29, 441–456.

Biziulevichius, G.A., Normantiene, T., 1997. Enchancement of lytic enzyme production resulting from replacement of the usual morphological variant of industrial lysosubtilin producer. Lett. Appl. Microbiol. 24, 133–135.

Biziulevichius, G.S., Kislukhina, O.V., Avizhienis, V.J., 1976. On the technology of receipt and specifity of action of the preparation lysosubtilin G 10×. Trudy VNII prikladnoi enzimologii (Proceedings of the All-Union Research Institute of Applied Enzymology 'Production and Application of Microbial Enzymatic Preparations') 3, 105–116 in Russian, with English abstract.

Biziulevichius, G.-A.S., Shablinskas, A.J., Zhukaite, V.P., Kislukhina, O.V., 1987. Lysosubtilin G 10×, a new antimicrobial agent for treatment and prophylaxis. Physicochemical properties. Antibiotiki i meditsinskaya biotekhnologiya (Antibiotics and Medicinal Biotechnology) 32, 331–333 in Russian, with English abstract.

Biziulevichius, G.-A.S., Shablinskas, A.J., Zhukaite, V.P., Kislukhina, O.V., 1989. Antibacterial spectrum of lysosubtilin G 10×. Antibiotiki i khimioterapiya (Antibiotics and Chemotherapy) 34, 579–581 in Russian, with English abstract.

Biziulevichius, G.A., Zhukaite, W., Kislukhina, O., 1995. Lysosubtilin – a broad-spectrum action preparation of lytic enzymes for veterinary use: in vitro studies on activators and inhibitors, complex structure and antifungal action. Biomed. Lett. 52, 43–57.

Blackburn, P., Pollack, J., 1988. Method of treating mastitis and other staphylococcal diseases. European Patent 359,873, 19 September.

Chadwick, D.J., Goode, J. (Eds.), 1997. Antibiotic Resistance: Origins, Evolution, Selection and Spread. Wiley, Chichester.

Chandler, D.S., Mynott, T.L., 1998. Bromelain protects piglets from diarrhoea caused by oral challenge with K 88 positive enterotoxigenic *Escherichia coli*. Gut 43, 196–202.

Chen, J.P., Chang, K.C., 1994. Immobilization of chitinase on a reversibly soluble-insoluble polymer for chitin hydrolysis. J. Chem. Tech. Biotechnol. 60, 133–140.

Chen, J.P., Chen, Y.C., 1997. Preparations of immobilized lysozyme with reversibly soluble polymer for hydrolysis of microbial cells. Bioresour. Technol. 60, 231–237.

Climo, M.W., Patron, R.L., Goldstein, B.P., Archer, G.L., 1998. Lysostaphin treatment of experimental methicillin-resistant *Staphylococcus aureus* aortic valve endocarditis. Antimicrob. Agents Chemother. 42, 1355–1360.

Crapisi, A., Lante, A., Pasini, G., Spettoli, P., 1993. Enchanced microbial cell lysis by the use of lysozyme immobilized on different carriers. Process Biochem. 28, 17–21.

Dean, C.R., Ward, O.P., 1991. Nature of *Escherichia coli* lysis by culture supernatants of *Bacillus* species. Appl. Environ. Microbiol. 57, 1893–1898.

Fraser, C.M., Bergeron, J.A., Mays, A., Aiello, S.E. (Eds.), 1991. The Merck Veterinary Manual. A Handbook of Diagnosis, Therapy, and Disease Prevention and Control for the Veterinarian, 7th ed. Merck, Rahway, NJ, pp. 181–184.

Fuglsang, C.C., Johansen, C., Christgau, S., Adler-Nissen, J., 1995. Antimicrobial enzymes: applications and future potential in the food industry. Trends Food Sci. Technol. 6, 390–396.

Ghuysen, J.M., Tipper, D.J., Strominger, J.L., 1966. Enzymes that degrade bacterial cell walls. Methods Enzymol. 8, 685–699.

Grenier, D., 1994. Effect of proteolytic enzymes on the lysis and growth of oral bacteria. Oral Microbiol. Immunol. 9, 224–228.

Guglielmo, B.J., 1995. Practical strategies for the appropriate use of antimicrobials. Pharm. World Sci. 17, 96–102.

Keck, G., Borne, P.M., 1995. Nouvelles conceptions en antibiothérapie et leurs applications pratiques en médecine vétérinaire. Rev. Méd. Vét. 146, 309–320.

Kislukhina, O.V., 1976. Determination of lytic activity of lysosubtilin by action towards *Escherichia coli* cells. Mikrobiologicheskaya promyshlennostj (Microbiol. Ind.) 141, 43–45 in Russian.

Kislukhina, O.V., Shevchuk, M.P., 1976. Bacteriolytic action of *Bac. subtilis* neutral proteinase. Mikrobiologicheskaya promyshlennostj (Microbiol. Ind.) 142, 10–12 in Russian.

Kleine, M.W., 1997. Introduction to oral enzyme therapy. Int. J. Immunother. 13, 59–65.

Kunin, C.M., 1993. Resistance to antimicrobial drugs – a worldwide calamity. Ann. Intern. Med. 118, 557–561.

Laskorin, B.N. (Ed.), 1989. Ion Exchanging Materials for Hydrometalurgy, Wastewater Treatment and Water Preparation Processes, A Reference Book, 4th ed. All-Union Research Institute of Chemical Technology, Moscow, pp. 42–43 in Russian.

Mayer, H., Viernstein, H., 1994. Herstellung magensaftresistent überzogener Enzympräparate für den Einsatz als Verdauungshilfen in der Tiernährung. Wien. Tierärztl. Mschr. 81, 103–107.

Moellering, R.C. Jr, 1998. Antibiotic resistance: lessons for the future. Clin. Infect. Dis. 27 (Suppl. 1), S135–S140.

Mynott, T.L., Chandler, D.S., Luke, R.K.J., 1991. Efficacy of enteric-coated protease in preventing attachment of enterotoxigenic *Escherichia coli* and diarheal disease in the RITARD model. Infect. Immun. 59, 3708–3714.

Mynott, T.L., Luke, R.K.J., Chandler, D.S., 1996. Oral administration of protease inhibits enterotoxigenic *Escherichia coli* receptor activity in piglet small intestine. Gut 38, 28–32.

Neu, H.C., 1992. The crisis in antibiotic resistance. Science 257, 1064–1073.

Samsonov, G.V., Kuznetsova, N.P., 1992. Crosslinked polyelectrolytes in biology. Adv. Polym. Sci. 104, 1–50.

Samsonov, G.V., Volskii, V.S., Shataeva, L.K., Zhukova, N.G., Zorina, A.I., Krylova, V.V., Laskorin, B.N., 1986. New types of high-permeable cation-exchangers for the biotechnology. Doklady Akademii Nauk SSSR (Proc. Acad. Sci. USSR) 288, 147–150 in Russian.

Sava, G., 1996. Pharmacological aspects and therapeutic applications of lysozymes. In: Jollès, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhauser, Basel, pp. 433–449.

Schulz, B., Schmidt, P.C., 1995. Enzymtableten-Fragen der Anwendung und Stabilität. Pharmazie 50, 519–524.

Severin, A., Tomasz, A., 1995. Selective lysis of cultures and cell walls of penicillin-resistant but not penicillin-susceptible *Streptococcus pneumoniae* strains by a murein hydrolase complex. J. Bacteriol. 177, 3316–3319.

Sisyagin, P.N., Zotkin, G.V., Redzhepova, G.R., Samsonov, G.V., Sochnev, V.V., Biziulevichius, G.-A.S., Urban, V.P., Yurchenko, V.S., Zhukova, N.G., Krylova, V.V., Dubinina, N.I., Ponomareva, R.B., Pashkovskaya, I.G., Zhukaite, V.P., Osipov, K.I., 1988a. Method of preventing dyspepsia and colibacillosis in calves. USSR Invention 1,624,743, 21 October.

Sisyagin, P.N., Zotkin, G.V., Urban, V.P., Redzhepova, G.R., Samsonov, G.V., Sochnev, V.V., Ponomareva, R.B., Kim, R.E., Yurchenko, V.S., Zhukova, N.G., Krylova, V.V., Dubinina, N.I., Biziulevichius, G.-A.S., Osipov, K.I., 1988b. Method of preventing dyspepsia and colibacillosis in calves. USSR Invention 1,622,985, 15 September.

Strominger, J.L., Ghuysen, J.M., 1967. Mechanisms of enzymatic bacteriolysis. Science 156, 213–221.

Thorne, K.J.I., Oliver, R.C., Barrett, A.J., 1976. Lysis and killing of bacteria by lysosomal proteinases. Infect. Immun. 14, 555–563.

Verhoef, J., 1994. Worldwide resistance against antibiotics. Int. J. Antimicrob. Agents 4, 141–142.

Zotkin, G.V., Samsonov, G.V., Biziulevichius, G.-A.S., Sisyagin, P.N., Urban, V.P., Yurchenko, V.S., Dubinina, N.I., Ponomareva, R.B., Pashkovskaya, I.G., Zhukaite, V.P., Osipov, K.I., Avzalov, F.Z., Idrisov, G.Z., Redzhep-

ova, G.R., 1987a. Method of treating gastrointestinal diseases in newborn calves using Fermosorb. USSR Invention 1,564,786, 23 December.

Zotkin, G.V., Sisyagin, P.N., Samsonov, G.V., Urban, V.P., Idrisov, G.Z., Yurchenko, V.S., Dubinina, N.I., Ponomareva, R.B., Biziulevichius, G.-A.S., Redzhepova, G.R., Borodacheva, O.V., Avzalov, F.Z., Osipov, K.I., 1987b. Method of treating gastrointestinal diseases in newborn calves using Polyferm. USSR Invention 1,557,720, 23 December.