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BIOSYNTHESIS AND SECRETION OF BOVINE GROWTH HORMONE IN Escherichia coli UNDER CONTROL OF A SECRETORY VECTOR CONTAINING PROMOTER AND SIGNAL SEQUENCE OF ALKALINE PHOSPHATASE GENE

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A recombinant plasmid was constructed containing the gene for bovine growth hormone joined with the regulatory region and the region coding the signal sequence of the Escherichia coli alkaline phosphatase gene. In conditions of phosphorus starvation, which causes derepression of alkaline phosphatase, expression was shown of the gene for bovine growth hormone, in addition to partial processing and secretion of protein into periplasm.

Advances in the study of recombinant DNA have created the possibility for expression of foreign genes in cells of microorganisms [1], which has expanded to a considerable degree the prospects for using microorganisms in biotechnology. However, during cytoplasmic expression of these genes, a number of difficulties have arisen associated with the fact that their products form insoluble aggregates and contain N-terminal methionine, and they also have low stability because of degradation under the influence of cytoplasmic proteases [2]. By constructing bacterial secretory vectors, not only has expression of foreign genes been accomplished in Escherichia coli, but also secretion of their products from cytoplasm into the periplasmic space or even into culture medium and, therefore, the disadvantages indicated can be avoided. It is known that secreted proteins of prokaryotes are synthesized in the form of precursors with an additional signal peptide 15-20 amino acids long on the N-end of the polypeptide chain. The signal peptide is absolutely necessary for secretion of protein through the plasma membrane and it is detached by signal protease on its outer side [3, 4]. Heterologous proteins are accurately processed and secreted, for example, in E. coli into the periplasmic space if they carry the bacterial signal peptide, although the level of their synthesis is often lower than during expression of genes with intracellular location of their products [1, 2]. Signal regions of genes of secreted proteins of E. coli such as β-lactamase [5]. OmpA protein [6-8], and thermostable enterotoxin II [9] are used as secretory vectors.

Very promising secretory vectors have also been created on the basis of the E. coli alkaline phosphatase gene. This enzyme is secreted into the periplasmic space [10], and its synthesis, in the same way as biosynthesis of other proteins of the pho regulon [11], is controlled by exogenous orthophosphate [12]. It is very important that the gene of this protein has the signal promoter [13, 14] and does not require expensive and not easily accessible promoters for expression. Using such vectors, the genes for human α-interferon [15], human growth hormone [13], and human epidermal growth factor [16] have been expressed and secretion of corresponding proteins into periplasmic space and accurate processing of their precursors have been demonstrated.

In the present work, construction of a vector which contains the promoter and signal region of the alkaline phosphatase gene is described, in addition to expression of the bovine growth hormone (bGH) in E. coli and distribution of synthesized protein among subcellular fractions.

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EXPERIMENTAL

Mutant strains *E. coli* C61 (phoS$^-$), *E. coli* LEP1 (phoB$^-$), and *E. coli* E15 (phoA$^-$) were used as the host cells. Cells of *E. coli* C61 constitutively synthesize alkaline phosphatase independently of the level of phosphate in medium as a result of disruption in the pst system of phosphate transport. The *E. coli* LEP1 and *E. coli* E15 mutants are negative for alkaline phosphatase because of a defect in the phoB gene of positive control and deletion of the phoA structural gene, respectively.

Cells were grown at 37°C overnight on medium containing 1% peptone (Chemapol), 0.5% yeast extract (Difco), and 1% NaCl. During culturing of cells with plasmids, 20 μg/ml ampicillin were added. Cells were collected by centrifugation, washed two times with 0.14 M NaCl, transferred into inorganic medium not containing orthophosphate [17], incubated 6.5 h at 37°C, centrifuged, and washed with 1 mM Tris-HCl buffer, pH 7.5.

Construction of AP2 Plasmid. Plasmid pHI-1 carrying the complete gene of *E. coli* alkaline phosphatase [18] was used as the source of promoter and the sequence coding the signal peptide of phoA. For this, pHI-1 DNA was cleaved with HindIII restrictase and a fragment 780 base pairs (b.p.) in size was isolated. Then, this fragment was cleaved with MspI restrictase and the ends formed were "blunted" with a Klenow fragment of *E. coli* DNA polymerase I and cleaved with HindIII restrictase. The mixture of restriction fragments was separated in 5% polyacrylamide gel and a fragment 350 b.p. in size was isolated (phoA fragment).

Plasmid pbGH18 containing full-sized cDNA of bovine growth hormone [19] was hydrolyzed by NarI restrictase, the 3' end was built up with a Klenow fragment, and again cleaved by BamHI. After electrophoresis in 5% polyacrylamide gel, a fragment ~720 b.p. in size was isolated (bGH fragment).

Both fragments were ligated to each other using phage T4 DNA ligase, then the ligase was inactivated, and the mixture was treated with HindIII and BamHI restrictases. After treatment with phenol and precipitation with alcohol, the mixture of fragments obtained was inserted into plasmid pBR322, treated with HindIII and BamHI restrictases, and the inner fragment was released.

Strain RR1 was used for transformation. The primary choice of clones was made according to phenotype Ap^RTc^S. Then, screening was conducted by hybridization of colonies [20] in parallel with two probes specific to the phoA section and to the section with the bGH gene, and clones were chosen which produced a positive response with both probes. Cleavage by restrictases, ligation of fragments, and transformation of *E. coli* were conducted according to the manual of Maniatis et al. [21].

Fractionation of Cells. Cells were treated with lysozyme (100 μg/ml) and EDTA (0.9 mM) in the presence of 0.5 M sucrose according to Miura and Mizushima [22]. Spheroplasts were removed by centrifugation at 16,000 rpm for 15 min; they were washed twice with 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, and 0.5 M sucrose. The fraction of periplasm obtained after precipitation of spheroplasts was dialyzed against water for a day and lyophilized, or concentrated by precipitation with cold acetone (−15°C). Washed spheroplasts were suspended in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride, and disintegrated with ultrasound on an apparatus from the MSE firm at a frequency of 20 kHz and an amplitude of 8 μm two times for 1 min each with an interval for precipitation. Intact cells were removed by centrifugation at 3000 rpm for 10 min, and functions of membranes and cytoplasm were dissociated by centrifugation at 100,000g for 40 min.

Identification of Growth Hormone. Cells or cell fractions were suspended in 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% Na dodecyl sulfate and 1% β-mercaptoethanol, incubated in a boiling water bath for 3 min, centrifuged, and supernatant liquid was used for analysis. Electrophoresis in the presence of 0.1% Na dodecyl sulfate was conducted according to Laemmli [23] in 12% polyacrylamide gel. After electrophoresis proteins were transferred onto BA-85 nitrocellulose (Schleicher und Schüll) by electroblotting for 1.5 h and bovine growth hormone was identified using immunoenzymatic analysis [24]. The nitrocellulose was washed for 5 min with TBS buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl), treated for 45 min with the same buffer containing 0.05% casein, and 0.01% Merthiolate (TBS-CM) to block free adsorption sites, incubated with rabbit antibodies to BGH (dilution of 1:1000), and washed five times for 5 min each with TBS buffer. Reaction to peroxidase ran at room temperature and with continuous mixing. A purified preparation of BGH synthesized in *E. coli* cells was used as the standard bovine growth hormone, which is comparable according to its properties with hypophyseal BGH.

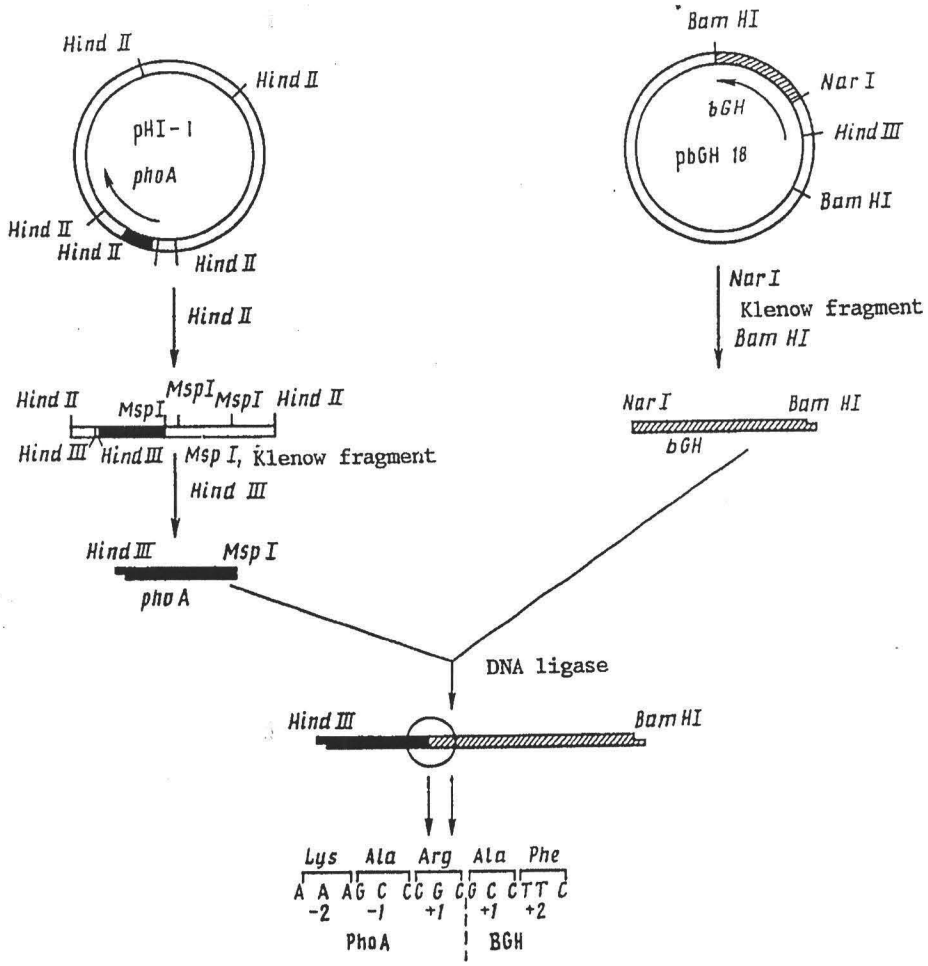

Fig. 1. Diagram of construction of hybrid gene containing promoter and signal sequence of alkaline phosphatase and bovine growth hormone.

Alkaline phosphatase activity was determined according to rate of hydrolysis of n-nitrophenyl phosphate by recording optical density at 400 nm [25].

Protein was determined according to Lowry [26].

RESULTS AND DISCUSSION

Construction of Plasmid Containing the bGH Gene under the Control of the Regulatory Region of the phoA Gene. The nucleotide sequence of the promoter and the region coding the NH_2 end of alkaline phosphatase are well known [27] and can be used both for study of the mechanism of protein secretion and for creation of secretory vectors to release foreign proteins into the periplasmic space of E. coli.

In the present work plasmid pHI-1 was used as the source of the promoter and sequence coding the signal peptide of alkaline phosphatase [18], from which the required HindIII-MspI fragment of DNA 350 b.p. in size was isolated using corresponding restrictases as described in the "Experimental" section. The foreign bovine growth hormone gene was isolated from plasmid pbGH18 [19] in the form of a NarI-BamHI fragment ~720 b.p. in size. Both fragments (phoA and bGH) were ligated to each other and inserted into plasmid pBR322 at the HindIII and BamHI sites with a defective gene for resistance to tetracycline. A diagram of the construction of the hybrid gene is presented in Fig. 1.

Recombinant plasmid AP2 therefore contains a hybrid gene which codes the signal sequence of alkaline phosphatase (21 amino acids) and mature BGH (191 amino acids). A feature of this hybrid gene is the presence of an insertion between these sequences which codes arginine, the N-terminal amino acid of mature alkaline phosphatase. Despite the presence of such an insertion, it could be expected that this hybrid gene can be expressed and its product secreted from cytoplasm.

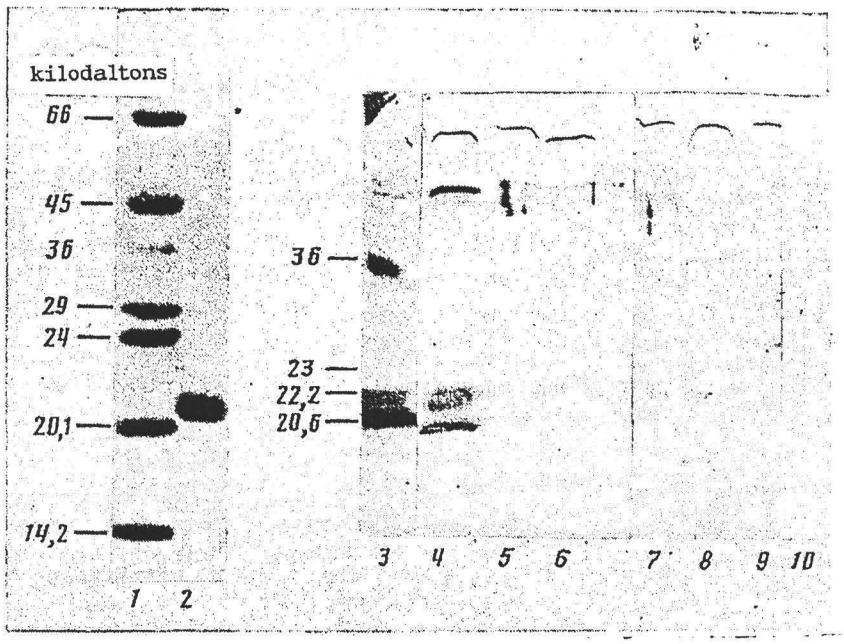

Fig. 2. Electrophoretic separation of bovine growth hormone synthesized in cells: 4, 9) E. coli AP2E15; 5, 7) E. coli AP2C61; 6, 8) E. coli AP2LEP1; 10) E. coli E15; growth on medium in the presence of phosphate (7, 8, 9) and after incubation in medium without phosphate (4, 5, 6, 10); 1) marker proteins (Sigma): bovine serum albumin (66 kilodaltons); yolk albumin (45 kilodaltons); glyceraldehyde-3-phosphate dehydrogenase (36 kilodaltons); carboanhydrase (29 kilodaltons); trypsinogen (24 kilodaltons); trypsin inhibitor from soybeans (20.1 kilodaltons); α-lactalbumin (14.2 kilodaltons) 2, 3) purified preparation of BGH (5 and 0.6 μg, respectively). Electrophoresis in 12% polyacrylamide gel in the presence of 0.1% Na dodecyl sulfate; 1, 2) staining with Coomassie R-250; 3-10) electroimmunoblotting with antibodies to BGH.

Expression of the bGH Gene in E. coli Cells. As is known, intensity of expression and secretion of foreign proteins is determined in a number of cases by the nature of the recipient strain. Thus, expression of epidermal growth factor under the control of the alkaline phosphatase gene increased by five times when a strain was used as host cells which does not synthesize alkaline phosphatase [16]. We also used strains as recipients which differ according to intensity of synthesis of alkaline phosphatase as a result of mutations in regulatory genes. In the E. coli constitutive strain C61, synthesis of enzyme does not depend on concentration of phosphate in medium. Cells of E. coli LEP1 and E. coli E15 do not synthesize enzyme. These strains were transformed by plasmid AP2 and biosynthesis and secretion of bovine growth hormone, and also proteins of the host cell secreted, were studied in the new transformant strains AP2C61, AP2LEP1, and AP2E15 as compared to the original strains.

Since expression of the alkaline phosphatase gene occurs in conditions of phosphorus starvation, synthesis of bovine growth hormone in strains AP2C61, AP2LEP1, AP2E15 transformed by plasmid AP2 was compared in conditions of phosphorus starvation. In order to identify BGH and reveal its location, electrophoresis was conducted in polyacrylamide gel in the presence of 0.1% Na dodecyl sulfate with subsequent immunoelectroblotting. The control preparation of BGH used by us, which was isolated from E. coli, appeared in the form of one band with a molecular weight of ~20.6 kilodaltons after electrophoresis and staining with Coomassie R-250, which apparently corresponds to mature protein (Fig. 2). Another molecular weight, 22 kilodaltons, has been presented in the literature for hypophyseal BGH [28], which may be associated either with the derivation of the preparation or with characteristics of the electrophoresis. Using immunoblotting, other components are detected (~22.2 and ~36 kilodaltons), in addition to the predominant protein with the same molecular

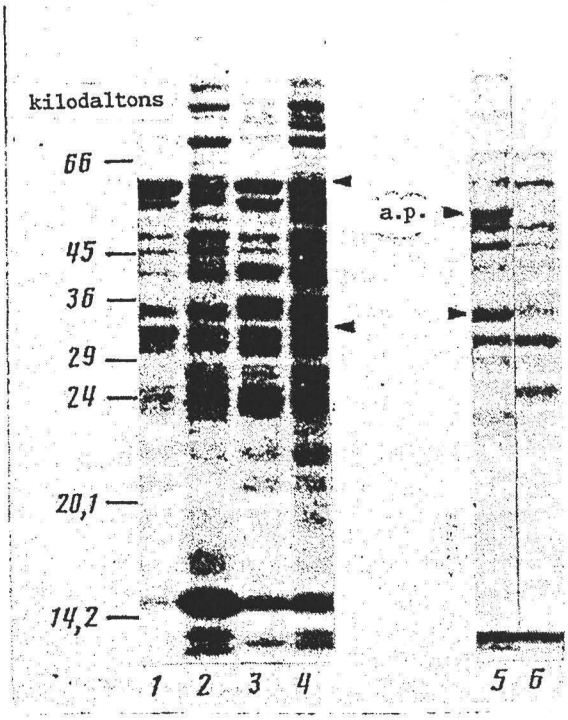

Fig. 3. Electrophoretic separation of periplasm proteins of transformed strains and recipient strains: 1) E. coli E15; 2) E. coli AP2E15; 3) E. coli LEP1; 4) E. coli AP2LEP1; 5) E. coli C61; 6) E. coli AP2C61. Cells were incubated in medium without phosphate. Electrophoresis in 10% polyacrylamide gel in the presence of 0.1% Na dodecyl sulfate; staining with Coomassie R-250.

weight (20.6 kilodaltons), which is present in minor amounts and revealed only immunologically. The presence of several forms is apparently the result of nonspecific modification and aggregation of protein. Such modifications were noted previously for human [13, 29] and bovine [28] growth hormones.

Analysis of the content of BGH in cells of strains AP2C61, AP2LEP1, and AP2E15 showed that its expression occurs only in strain AP2E15 (Fig. 2), which does not synthesize alkaline phosphatase because of deletion of the PhoA structural gene, and only in conditions of phosphorus starvation. In this strain, in addition to components corresponding in mobility to those of the control preparation, and thus to mature protein, one other component is found with a molecular weight of ~23 kilodaltons. The fact that these are specific immune complexes of BGH is indicated by their absence in E. coli E15 host cells. The protein with a molecular weight of ~23 kilodaltons is apparently a hybrid protein containing the complete amino acid sequence of the mature bovine growth hormone and the signal peptide of alkaline phosphatase, since it differs from it by a fragment with a molecular weight of ~2.4 kilodaltons, which corresponds to the size of the signal peptide of alkaline phosphatase. This hybrid protein is analogous in structure to precursors of secretory proteins.

Therefore, results attest to the fact that synthesis of hybrid BGH and partial processing of it with detachment of the peptide occur in plasmid strain AP2E15.

In order to clarify whether biosynthesis of foreign protein and the presence of plasmid influence synthesis of inherent proteins of the host cell, a comparison was made of the protein composition of periplasm fractions, membranes, and cytoplasm of host cells with those in the three transformed strains AP2E15, AP2C61, and AP2LEP1 (Fig. 3). In strain AP2E15, in which expression of the bGH gene is noted in conditions of phosphorus starvation, decreased synthesis and secretion of some periplasm proteins is noted (Fig. 3, lanes 1, 2). However, an analogous situation is also noted in AP2C61 cells, although in this strain no expression of BGH is detected. One of these proteins in strain AP2C61 is alkaline phosphatase Fig. 3, lanes 5, 6). Decreased content of enzyme by 90% is also corroborated by enzymatic analysis of periplasm of strain AP2C61. Reduced content of these proteins,

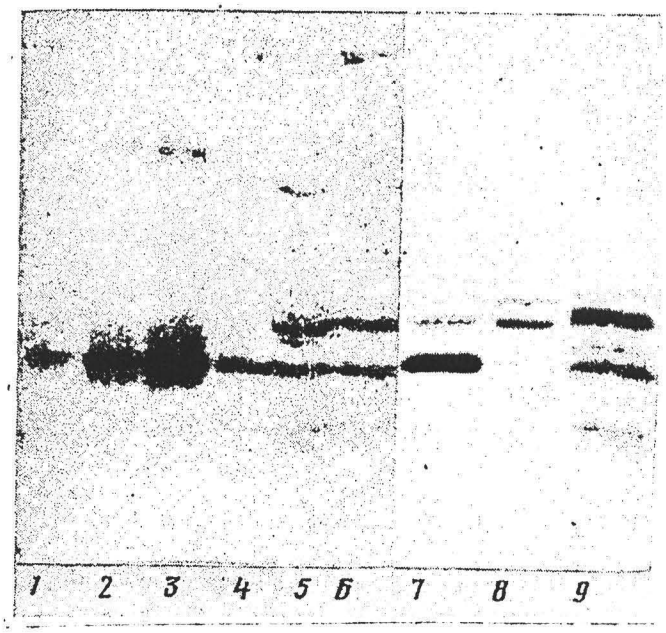

Fig. 4. Electrophoretic separation of bovine growth hormone contained in different fractions of E. coli AP2E15 cells: 4, 7) in periplasm; 5, 8) in cytoplasm; 6, 9) in membranes; 4, 6) isolated from equal numbers of cells; 7-9) samples with different protein content; 1-3) 0.12, 0.24, and 0.6 μg purified preparation of BGH, respectively. Electrophoresis in 12% polyacrylamide gel in the presence of 0.1% Na dodecyl sulfate. The BGH was revealed by electroimmunoblotting with antibodies to this protein.

which is not dependent on biosynthesis of the foreign protein, the growth hormone, permits it to be thought that it is not expression of the foreign gene which influences biosynthesis of proteins of the host cell, but some other factors. At the same time, the influence of the plasmid can not be suggested, since transformation of strain LEP1 by it does not lead to suppression of synthesis of any proteins of the host cell. In all transformed strains, changes are also not noted in content of membrane and cytoplasmic proteins (data not presented). The appearance of a large amount of protein with a molecular weight of 14.5 kilodaltons in periplasm of plasmid strains AP2E15 and AP2LEP1 should be noted.

Therefore, it is difficult to make a final conclusion as to whether expression of foreign genes and secretion of their products compete with biosynthesis and secretion of proteins of the host cell.

Secretion of BGH into Periplasm of E. coli AP2E15 Cells. It is known that processing of the precursor of secreted protein occurs after protein moves through the cytoplasmic membrane onto its outer side. The presence of two forms of BGH in E. coli AP2E15 cells permits the suggestion that synthesized foreign protein is either located in different compartments or some protein is not processed during secretion. In order to establish the location of forms of BGH revealed, fractionation of cells was conducted with the derivation of fractions of periplasmic material, membranes, and cytoplasm. Content of BGH in each fraction was analyzed using immunoelectroblotting (Fig. 4), and estimated quantitatively by scanning of immunoblots (Table 1).

Results presented in Fig. 4 (lanes 4-6) and Table 1 reflect the ratios of both forms of BGH in different cellular fractions. As follows from Fig. 4 and Table 1, the two forms of BGH found during analysis of lysates of whole cells are also found in cellular fractions isolated. One of them has a molecular weight coinciding precisely with that of mature protein and therefore represents the processed mature form of BGH (~60% of total amount of BGH synthesized in cells). Processed BGH, as should be expected, is found in the periplasmic space (Fig. 4, lanes 4 and 7, Table 1), which attests to secretion and accurate processing of bovine growth hormone in E. coli cells. In special control experiments it was shown that the presence of this form in the fraction of periplasmic material is not the result of

TABLE 1. Biosynthesis and Location of Bovine Growth Hormone in E. coli AP2E15 Cells

Fraction of cells	Content of bovine growth hormone (BGH)				
	μg			%	
	in 1 liter of culture	in 1 mg total protein of cell	in 1 mg protein of fraction	proportion of amount of individual form	proportion of total amount of BGH
	Hormone processed (20.6 kilodaltons)				
Whole cells	309,6				58,9
Periplasm	142,2	0,17	2,91	46	27,1
Cytoplasm	95,4	0,11	0,13	31	18,2
Membrane	72	0,07	0,97	23	13,6
	Precursor (23.0 kilodaltons)				
Whole cells	216,0				41,1
Periplasm	–	–	–	–	–
Cytoplasm	154,8	0,19	0,21	71,6	29,5
Membrane	61,2	0,09	1,14	28,3	11,6

lysis of spheroplasts, since no activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase was noted in this fraction. However, a substantial amount of processed BGH (~50%) is distributed between cytoplasm and membranes. This is apparently the result of contamination of these fractions by material of the periplasm, and is also possibly the result of processing of the precursor with disintegration of spheroplasts and their fractionation. Analysis of equal amounts (by protein) of fractions showed that specific content of BGH in periplasm exceeds by approximately 14 times that in cytoplasm (Table 1, Fig. 4).

As far as the unprocessed form of protein is concerned, i.e., its precursor, it constituted ~40% of total growth hormone synthesized. A large amount of this form (~70%) is located in cytoplasm and only ~30% in membranes. In the fraction of periplasmic material, this form of protein is almost absent.

The comparatively large amount of precursor protein attests to some kind of restrictions impeding its processing and secretion. As is known, secondary structure of the region of proteolytic cleavage is very important for processing [30]. The hybrid protein studied by us has a section of processing which differs in structure from those for alkaline phosphatase or bovine growth hormone, since there is an Arg insertion between the signal sequence of alkaline phosphatase and mature BGH. In mature alkaline phosphatase the first amino acid is also Arg; however, the next amino acids to it are Thr-Pro- [27], while the N-terminal amino acids of mature BGH are Ala-Phe-. Therefore, differences in the immediate vicinity of the intact section, at which detachment of the signal peptide from alkaline phosphatase occurs, apparently impedes the optimal effect of the signal peptidase of E. coli, which makes processing of the hybrid protein difficult. It is known that a specific number of secretion sites exists in the membrane [31]. Therefore, inhibited processing can lead to blocking of secretion sites and as a result, to decrease in level of expression of hybrid gene bGH and accumulation of unprocessed protein in cytoplasm.

When estimating the overall level of expression of the bGH gene in E. coli AP2E15 cells, it can be noted that it is slightly lower than for other proteins in E. coli cells when a secretory vector is used based on the gene for alkaline phosphatase, and constitutes ~500 μg/liter of cell culture (~125 ng per 1 unit at A_{600}, i.e., 0.6% of the total amount of cellular protein). At the same time yield of human epidermal growth factor in analogous conditions of culturing cells constituted 1000 μg/liter of culture [16], while human growth hormone is 280 ng per 1 unit at A_{600} [13]. However, it should be noted that different methods were employed in these works for quantitative estimation of the yield of protein.

Therefore, using a secretory vector containing regulatory and signal regions of the alkaline phosphatase gene, expression of the bGH gene was achieved, in addition to partial secretion of protein. Secretion of protein into the periplasmic space, where its specific content is ~14 times higher than within the cell, and its accurate processing create the prerequisites for a simpler system of isolation and purification of BGH synthesized by E. coli cells. The next task consists of increasing the total yield of protein both by changing construction of the hybrid gene and by choosing more optimal conditions of culturing producer and expression of the hybrid gene [16, 31].

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