Biochemistry (Moscow), Vol. 65, No. 12, 2000, pp. 1397-1402. Translated from Biokhimiya, Vol. 65, No. 12, 2000, pp. 1659-1665.

Preparation and Properties of *Clostridium thermocellum* Lichenase Deletion Variants and Their Use for Construction of Bifunctional Hybrid Proteins

K. A. Musiychuk*, I. V. Goldenkova, R. M. Abdeev, N. S. Kobets, and E. S. Piruzian

Vavilov Institute of General Genetics, Russian Academy of Sciences, ul. Gubkina 3, Moscow, 117809 Russia; fax: (095) 132-8962; E-mail: kostyam@vigg.ru

Received April 10, 2000
Revision received June 14, 2000

Abstract—Major properties (pH and temperature optimum, stability) of lichenase (β-1,3-1,4-glucanase) deletion variants from *Clostridium thermocellum* were comparatively studied. The deletion variant LicBM2 was used to create hybrid bifunctional proteins by fusion with sequences of the green fluorescent protein (GFP) from *Aequorea victoria*. The data show that in hybrid proteins both GFP and lichenase retain their major properties, namely, GFP remains a fluorescent protein and the lichenase retains activity and high thermostability. Based on the results of this investigation and results that have been obtained earlier, the use of the deletion variants of lichenase and the bifunctional hybrid proteins as reporter proteins is suggested.

Key words: thermostable lichenase (β-1,3-1,4-glucanase), green fluorescent protein (GFP), reporter systems, hybrid bifunctional proteins

Some organisms such as the cellulolytic anaerobic bacteria *Ruminococcus albus*, *Clostridium cellulolyticum*, and *Clostridium thermocellum*, as well as the fungal pathogen *Trichoderma reesei*, possess complicated multienzyme complexes referred to as cellulosomes [1, 2]. They usually include different enzymes from the family of glycosyl hydrolases (glucanase, glucosidase, xylanase, cellulase) and scaffolding proteins that support the structural integrity of the cellulosome. All cellulosomal proteins have a particular domain in their structure that is joined with the scaffolding protein of the cellulosome [3].

Previously in our laboratory, the gene of *lic*B encoding β-1,3-1,4-glucanase (lichenase) (endo-β-1,3;1,4-glucan-D-glycosylhydrolase, EC 3.2.1.73 (P29716)) of *Clostridium thermocellum* was cloned [4]. The nucleotide sequence of *lic*B gene was determined and properties of the enzyme were studied [5-7].

Under natural conditions, native lichenase (LicB) is included in the cellulosomal complex and hydrolyzes endo-β-1,3-glucoside bonds in polyglucans with mixed endo-β-1,3-1,4-glucoside bonds [5].

Like most glycosylhydrolases, lichenase is a single subunit protein consisting of several structural domains: the N-terminal signal peptide (27 amino acid residues), the catalytic region (224 amino acid residues), a segment enriched with Pro and Thr residues (Pro-Thr-sequence, 13 amino acid residues), and the cellulosome-binding domain (CBD, 65 amino acid residues). The Pro-Thr-sequence is a flexible linker that usually joins domains. The cellulosome-binding domain includes two conservative repeats of 25 amino acid residues each [4]. Similar domains are found in all cellulosomal proteins (usually in the C-terminus) [3].

Thermostable enzymes are widely used in biotechnology [8] and as models for investigation of problems of protein folding and for determination of mechanisms of thermostability [9].

The high activity and thermostability of the LicB bacterial enzyme from *C. thermocellum* is used as the basis for the development and proposal of a new reporter system [10, 11].

Reporter systems are widely used for studying the regulation of gene expression [12-15]. There are particular requirements for a reporter system [12], e.g., size of the gene and its product and the ability to maintain the reporter enzyme activity when it is fused to other proteins at its terminuses. Several reporter systems are now widely used [13-15]. For example, a reporter system of the green

* To whom correspondence should be addressed.

0006-2979/00/6512-1397$25.00 ©2001 MAIK "Nauka/Interperiodica"

fluorescent protein (GFP) of *Aequorea victoria* allows real-time monitoring of gene expression (*in vivo*) in a single cell [16]. All well-known reporter systems have some disadvantages [12-14] that limit their application to some investigations. It is difficult to expect that ideal reporter systems will be found. However, looking for new reporter systems [16, 17] and modification of presently used reporter systems are promising tasks [18, 19]. Modification of existing reporter systems can lead to the creation of bifunctional reporter systems. This approach makes it possible to combine the advantages of two reporter systems or to compensate for their disadvantages [18].

Our investigation is devoted to modification of a thermostable lichenase, namely, to obtaining deletion variants of the *C. thermocellum* lichenase and studying of its properties as well as for creating hybrid bifunctional proteins LicB-GFP and GFP-LicB. The deletion variants of the lichenase and the hybrid bifunctional proteins can be suggested for use as reporters.

MATERIALS AND METHODS

Construction of plasmids. To obtain deletion variants of the *lic*B gene, previously described plasmids pUC42-H [20] and pRT3 [11] were used, and for the native protein plasmid pCU403 was used [7]. As a source of the *gfp* gene of *Aequorea victoria* the plasmid pAVA120 was used [21]. The standard procedures for molecular cloning and PCR protocols were used [22]. Restriction endonucleases, T4 DNA ligase, DNA polymerase I (Klenow fragment), and alkaline phosphatase were used as recommended by the manufacturers (Promega, USA; Fermentas, Lithuania). To express the deletion variants of the *lic*B gene in *E. coli* cells and for its purification, pQE vectors (QIAexpress, USA) [23] were used. The pQE expression vectors contain the sequence encoding the 6*His which allows purifying of recombinant protein by affinity chromatography on Ni-NTA-agarose.

To construct the pQE-licBM1 vector, the *Bam*HI-*Pst*I fragment of the pUC42-H plasmid was cloned in the pQE-30 vector. The pQE-licBM2 vector was constructed in several steps. First, the licBM2 sequence was obtained by PCR using pRT3 plasmid as the template and primers: 5′-gaaagcataacacaacaadgcc-3′ and 5′-tcggagtagaaggagcaatcg-3′ (Syntol, Russia). The PCR fragment obtained was cloned in the pT7blue plasmid (Novagen, USA) to create the pTLU and pTLD plasmids that have opposite orientations of the fragment. Further, the *Bam*HI-*Pst*I fragment containing the sequence of licBM2 from pTLU plasmid was cloned in the pQE-30 vector to construct the pQE-licBM2 plasmid.

To construct the pQE-gfp vector, the *Nco*I-*Xba*I fragment containing the *gfp* gene from the pAVA120 plasmid was inserted into the pRT100 [24] plasmid to create pRT-gfp. The *Xho*I-*Hind*III fragment from pRT-gfp was inserted into pQE-30 (previously digested by *Nco*I and then by Klenow fragment) between the *Sal*I and *Hind*III sites to construct pQE-gfp.

To create pQE-gfp-licBM2 and pQE-licBM2-gfp plasmids, several intermediate steps were used. First, the sequence of *lic*BM2 was isolated using PCR and pQE-licBM2 as a template and primers 5′-caaccatggtaaatacgcct-3′ and 5′-tagcagatctcggagtagaaggagc-3′ (Syntol) using PCR. The PCR fragment was digested with *Nco*I and *Bgl*II and inserted into pQE-gfp to create pQE-licBM2-KM2-Mys25. The *Bam*HI-*Bgl*II fragment of this plasmid was cloned in pQE-gfp digested by *Bam*HI to construct pQE-gfp-licBM2. The *Bam*HI-*Hind*III fragment from pQE-licBM2-KM2-Mys25 was inserted into pQE-gfp digested with *Bgl*II and *Hind*III to create pQE-licBM2-gfp.

Preparation and purification of protein extracts. Cultivation of *E. coli* cells (strain XL1-blue), previously transformed by plasmids (pCU403, pUC42-H, pQE-licBM1, pQE-licBM2, pQE-gfp, pQE-gfp-licBM2, and pQE-licBM2-gfp) and induction analysis were done according by the protocols of QIAexpress. The bacterial extracts for testing activity of the enzyme were obtained by cell lysis in 8 M urea followed by centrifugation (2000*g*, Beckman J2-21, rotor Ja20, 30 min) and dialysis against 50 mM Tris-HCl, pH 8.0.

The activity of lichenase was estimated using lichenan (Sigma, USA) as the substrate. Reducing sugars released from the substrate were determined by the method of Wood and Bhat [25] using a calibration scale established with glucose. One unit of activity is defined as the quantity of enzyme releasing 1 μmol of reducing sugars (as glucose equivalent) per minute.

Protein was measured according to Bradford [26] using the dye reagent (BioRad, USA) and bovine serum albumin as standard (Sigma).

Electrophoresis of proteins was performed in a denaturing polyacrylamide gel according to Laemmli [27].

Zymograms were obtained by staining of lichenan (0.1%) containing gel according to Teather and Wood [28], with several modifications. Proteins were separated by electrophoresis in a 10% or gradient 8-16% polyacrylamide gel in the presence of SDS. Lichenan was added to the separating gel before polymerization. Before application the sample, loading buffer was added to bacterial extracts (1-10 μg) and the samples were incubated at 70°C for 40 min.

Testing of the lichenase activity on plates was done according to Beguin [29]. Colonies of the *E. coli* were grown on agar (1.5%) medium at 37°C overnight. Then the colonies were covered by top agar (0.8% agarose) containing 0.05% lichenan. The plates were exposed at 65°C for 1-3 h. The plates were stained with 0.5% Congo Red for 15 min. The unbound dyestuff was washed off with 1 M NaCl solution.

The enzyme was purified on Ni-NTA resin according to protocols of the manufactures (QIAGEN, GmbH, Germany). NTA resin is a chelating adsorbent having four chelating sites that can interact with Ni^{2+}. Thus NTA occupies four of the six ligand binding sites in the coordination sphere of the Ni^{2+}, leaving two sites free to interact with the 6*His tag placed at the N-terminus of the GFP-LicB protein.

Fluorescence of GFP was observed under UV-irradiation and pictures were taken with a digital camera (Olympus C-1400XL, Japan).

RESULTS AND DISCUSSION

By modification of the nucleotide sequences as described in "Materials and Methods", the *C. thermocellum* lichenase deletion variants LicBM1 and LicBM2 were obtained.

The 31 N-terminal amino acid residues and 31 C-terminal amino acid residues have been cut off to create LicBM1. The first sequence is typical for leader signals [6], and the second carrying one of the conservative repeats of the cellulosome-binding domain is typical of cellulosomal proteins [5, 6].

Another deletion variant LicBM2 contains only the sequence of the catalytic domain and a Pro-Thr-sequence resulting from truncation of the 40 C-terminal amino acid residues including the second conservative repeat of the cellulosome-binding domain.

Removal of the leader sequence from the N-terminus of the enzyme should not affect the enzyme activity because in *C. thermocellum* the native protein is secreted to the intercellular space and the leader peptide is cleaved by a leader peptidase [6].

As to partial (LicBM1) or complete (LicBM2) deletion of the cellulosome-binding domain, it should be pointed out that these domains are not directly involved in catalytic function, but they might change the activity of the catalytic domains [3, 30].

The deletion variants of lichenase were analyzed using zymograms. The bands of activity LicBM1 and LicBM2 agree with theoretical computed molecular masses of the modified enzymes, 33 and 28 kD, respectively (Fig. 1). Additional bands of activity are likely to appear due to alternative translation start or retardation of lichenase in gel containing lichenan [7].

To determine whether the modification of the enzymes influenced their properties, the pH optimum, temperature optimum, and thermostability of the deletion variants were comparatively investigated (Figs. 2 and 3).

The curves of dependence the relative activity of the variants on pH have similar form for all variants of the protein. The enzyme retains 80% activity over a wide range of pH values (6.0-9.5), and the pH optimum is at 8.0. However, the deletion variants show lesser activity at acid pH (Fig. 2a).

The curves of dependence of the relative activities of the different variants on temperature are presented in Fig. 2b. The temperature optima have only slightly changed in the deletion variants, being 65°C in comparison with 70°C for the native protein. It should be pointed out that if 0.1 mg/ml BSA is added in reaction mixture, the temperature optima became 70°C for both deletion variants (data not shown). These results can be explained by measuring of activity at high dilution level of the protein, while in the presence of BSA the protein is stabilized.

The results of analysis of the stabilities of deletion variants without substrate at different temperatures are presented in Fig. 3. Based on the results obtained, it is seen that the stability of the deletion variants is not changed. However, at the temperature optimum (65°C) LicBM1 and LicBM2 retain activity longer than native protein does. Therefore, using the deletion variants one can test a small amount of protein while increasing the incubation time at the optimum temperature.

In summary, partial or full deletion of the C-terminal domain does not lead to major changes in the enzymatic properties. The lichenase deletion variants retain the temperature optimum, are active over a wide range of pH with maximum at pH 8.0, endure long incubation at 65°C (more than 4 h), and remain active after multiple freeze–thaw cycles of the samples (data not shown). It is

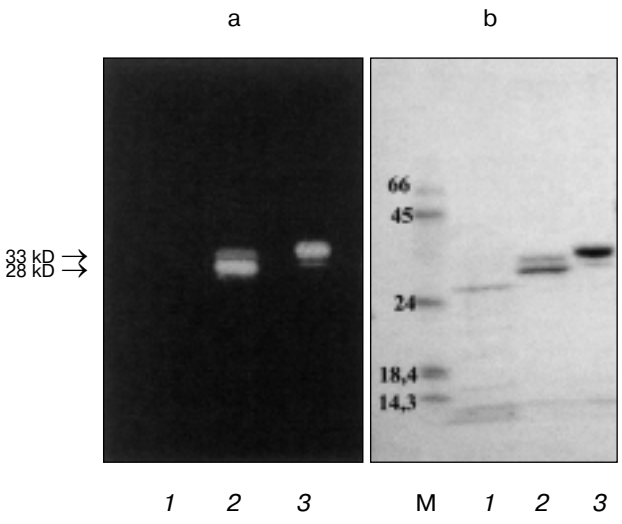

Fig. 1. Zymogram (a) and electrophoregram (b) of bacterial protein extracts in the presence of 0.1% lichenan as a substrate. Lanes: *1*) control; *2*) LicBM2; *3*) LicBM1; M, molecular weight markers. Size markers (in kD) used were: 66, bovine albumin; 45, egg albumin; 24, trypsinogen; 18.4, β-lactoglobulin; 14.3, lysozyme (Sigma, USA). As a control, bacterial protein extract from a strain carrying pQE-30 was used.

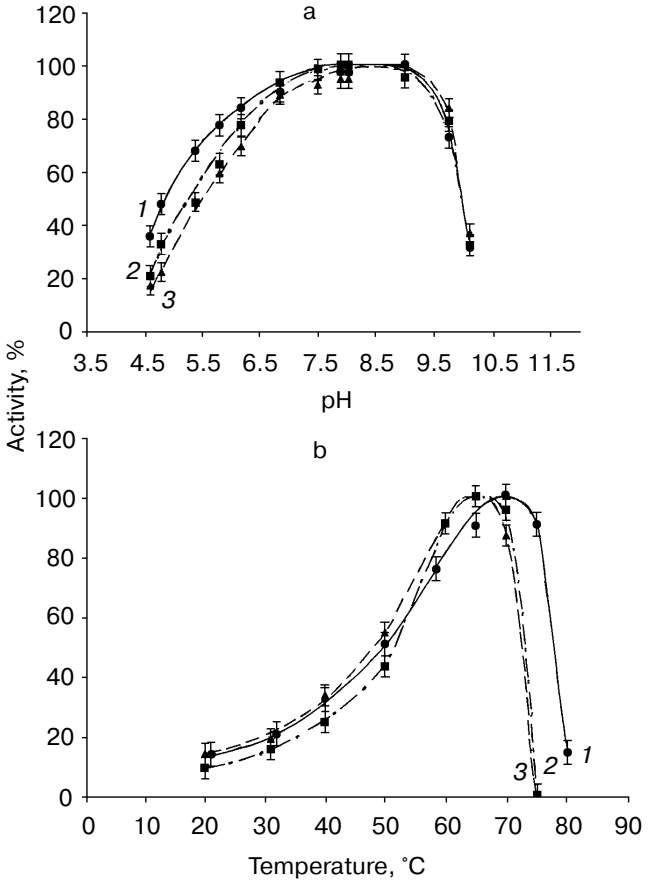

Fig. 2. Functional dependence of the activity of lichenase deletion variants on pH (a) and temperature (b): *1*) native enzyme; *2*) deletion variant LicBM1; *3*) deletion variant LicBM2.

important that the deletion variants are smaller. For further investigations, the deletion variant LicBM2 was chosen.

The choice of the LicBM2 deletion variant for further investigations was based on the fact that LicBM2 retains the main properties of the enzyme (activity and thermostability) and has lower molecular weight (28 kD) in comparison with LicBM1 (33 kD) and the full protein (36 kD). In addition, it should be emphasized that the full deletion of the C-terminal CBD allows doing fusions of the LicBM2 deletion variant through the linker sequence of the Pro-Thr-box with other sequences. The is important for the subsequent use of lichenase as a reporter protein.

Each reporter system has advantages and disadvantages. The main disadvantage of the lichenase reporter protein is the impossibility of determination of its activity *in vivo* and/or *in situ* [14]. At the same time, the great advantage of GFP is its fluorescence. This feature allows testing of its activity without substrates, cofactors, or disruption of the living cell [11-14]. However, the accurate quantitative measurement of GFP fluorescence is complicated [13, 15], whereas the lichenase possesses exact, fast, and highly sensitive methods for determination of activity that do not need special and costly facilities [10, 11, 14]. To solve these problems of the reporter systems, bifunctional reporter systems in which advantages of one reporter system compensate for disadvantages of another can be constructed [18].

The possibility of making such bifunctional proteins has been shown in several studies [18, 31-33]. Bifunctional enzymes are found among the glycosylhydrolases, which possess substrate specificity inherent to

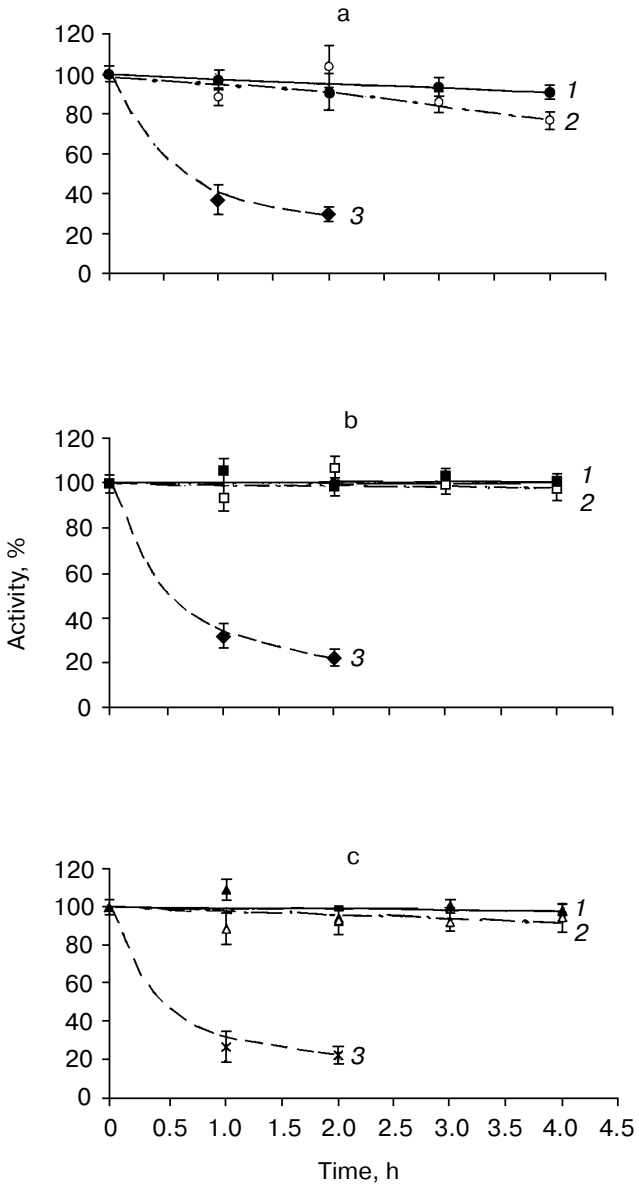

Fig. 3. Stability of the lichenase deletion variants at 65 (*1*), 70 (*2*), and 85°C (*3*). a) Native enzyme; b) deletion variant LicBM1; c) deletion variant LicBM2.

different families of glycosylhydrolases. This is due to the presence of two catalytic domains [31, 32]. Recently a hybrid multienzyme has been produced—the product of expression of a chimeric gene obtained by fusion of cellulase of *Erwinia carotovora* and a thermostable β-1,3;1,4-glucanase of *Bacillus*. Expression of the chimeric gene in yeast cells leads to the formation of a multienzyme having two catalytic domains with different substrate specificities and connected by a linker sequence. This enzyme is active toward a soluble derivative of cellulose and toward the β-1,3;1,4-glucan of oats. The levels of its activities were significantly higher than that of the native enzymes [33]. Through the fusion of the genes encoding GFP and *E. coli* β-glucuronidase, bifunctional reporter constructs were obtained for investigating plant gene expression [18]. The results mentioned above suggest that similar fusion of proteins to produce bifunctional multienzymes can be regarded as an effective method for creating improved enzymes for biotechnology and for basic investigations.

As a result of the molecular cloning described in "Materials and Methods", vectors have been obtained where the sequences encoding GFP and LicBM2 were fused in frame with the production of hybrid proteins: LicBM2-GFP and GFP-LicBM2. The expression of the resulting chimeric sequences in *E. coli* has demonstrated the formation of the bifunctional proteins. The results of the plate test (Fig. 4) indicate that in the hybrid proteins both GFP and lichenase retain their main properties, namely fluorescence for GFP (Fig. 4b) and activity at high temperature (65°C) for lichenase (Fig. 4a).

The hybrid proteins LicBM2-GFP and GFP-LicBM2 before and after purification by affinity chromatography were analyzed by zymograms (Fig. 5). The apparent molecular masses of the bands showing hybrid protein activity agreed with the theoretically predicted for chimeric proteins GFP-LicBM2 (56 kD) and LicBM2-GFP (56 kD). The additional bands of activity on the zymogram can be explained by proteolysis of the hybrid proteins. It should be noted that together with the possibilities of qualitative testing of lichenase activity, the zymogram method allowed the determination of the molecular masses of the hybrid proteins carrying the lichenase. Therefore, using the lichenase as a reporter protein in the zymogram method can be suggested as an alternative to Western-blot hybridization.

This investigation has also shown that the deletion variants of lichenase (LicBM2) tolerate significant fusion at both the C-terminus and at the N-terminus while retaining its activity. As mentioned above, this property is very important for reporter proteins.

Thus, the results of this investigation show that removal of the leader sequence and partial (LicBM1) or full (LicBM2) deletions of the cellulosome-binding domain lead to no significant changes of the main properties of the enzyme: pH and temperature optima, activity, and stability. In some cases (as mentioned above), the deletion variant LicBM2 has advantages for using it as a reporter protein and for the production of hybrid bifunctional proteins. The data concerning the retention of the main properties of the lichenase (activity) and GFP (fluorescence) in the bifunctional hybrid proteins suggest its use as a bifunctional reporter for studying of gene expression regulation.

This work was given financial support by the Russian Foundation for Basic Research, grant No. 98-04-48670.

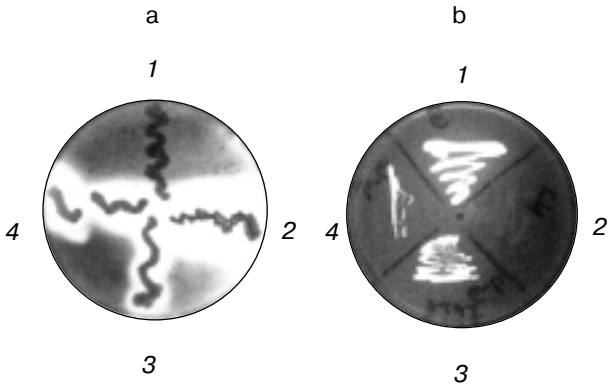

Fig. 4. Plate tests of the lichenase activity (a) and GFP fluorescence (b) of hybrid proteins GFP-LicBM2 and LicBM2-GFP. Strain *E. coli* transformed by plasmids pQE-gfp (*1*), pQE-licBM2 (*2*), pQE-gfp-licBM2 (*3*), and pQE-licBM2-gfp (*4*).

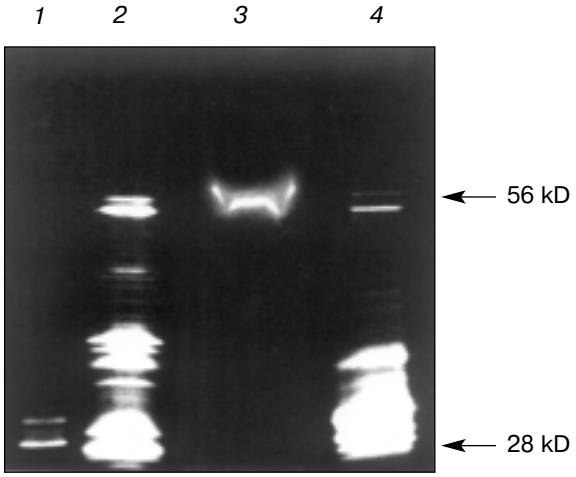

Fig. 5. Zymogram of bacterial protein extracts with 0.1% lichenan as a substrate. Lanes: *1*) LicBM2; *2*) GFP-LicBM2; *3*) GFP-LicBM2 (after purification by affinity chromatography); *4*) LicBM2-GFP. Arrows show the bands of lichenase activity. Molecular weights of the hybrid proteins and recombinant lichenase are marked. The molecular weight of native GFP is 28 kD.

REFERENCES

1. Sprey, B., and Lambert, C. (1983) *FEMS Microbiol. Lett.*, **18**, 217-222.
2. Coughlan, M. P., and Ljunghahl, L. G. (1988) *In Biochemistry and Genetics of Cellulose Degradation, FEMS Symposium*, Academic Press, **43**, 11-30.
3. Bayer, E. A., Chanzy, H., Lamed, R., and Shoham, Y. (1998) *Curr. Opin. Struct. Biol.*, **8**, 548-557.
4. Piruzian, E. S., Mogutov, M. A., Velikodvorskaya, G. A., and Akimenko, B. K. (1985) *Dokl. AN SSSR*, **281**, 963-965.
5. Schimming, S., Schwarz, W. H., and Staudenbauer, W. L. (1991) *Biochem. Biophys. Res. Commun.*, **177**, 447-452.
6. Schimming, S., Schwarz, W. H., and Staudenbauer, W. L. (1992) *Eur. J. Biochem.*, **204**, 13-19.
7. Zverlov, V. V. (1993) *Study of structure of the lichenase gene of Clostridium thermocellum F7 and characterization of its product*: Candidate's dissertation [in Russian], IMG RAN, Moscow.
8. Hagerdal, B., Ferrchak, J., and Pye, E. (1980) *Biotech. Bioeng.*, **12**, 1515-1526.
9. Jaenicke, R. (1998) *Biochemistry (Moscow)*, **63**, 312-321.
10. Piruzian, E. S., Shigapova, N. V., Kobets, Y. S., Goldenkova, I. V., and Los, D. A., *Patent No. 2147035.*
11. Piruzian, E. S., Monzavi-Karbassi, B., Darbinian, N. S., Goldenkova, I. V., Kobets, N. S., and Mochulsky, A. V. (1998) *Mol. Gen. Genet.*, **257**, 561-567.
12. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) *EMBO J.*, **6**, 3901-3907.
13. Rutter, G. A., Kennedy, H. J., Wood, C. D., White, M. R. H., and Tavare, J. M. (1998) *Chemistry and Biology*, **5**, R285-R290.
14. Piruzian, E. S., Goldenkova, I. V., Musiychuk, K. A., Abdeev, R. M., Volkova, L. V., and Kobets, N. S. (2000) *Fiziol. Rast.*, **37**, 382-389.
15. Naylor, L. H. (1999) *Biochem. Pharmacol.*, **58**, 749-757.
16. Chalfie, M. Tu. Y., Euskrichen, G., Ward, W. W., and Prasher, D. C. (1994) *Science*, **263**, 802-805.
17. Wildt, S., and Deuschle, U. (1999) *Nature Biotech.*, **17**, 1175-1178.
18. Quaedvlieg, N. E. M., Schlaman, H. R. M., Admiraal, P. C., Wijting, S. E., Stougaard, J., and Spaink, H. P. (1998) *Plant Mol. Biol.*, **38**, 861-873.
19. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) *Trends Biochem. Sci.*, **20**, 448-455.
20. Darbinian, N. S., Popov, Yu. G., Mochulskiy, A. V., Volkova, L. V., Piruzian, E. S., and Vasilevko, V. T. (1996) *Genetika*, **32**, 197-203.
21. Von Arnim, A. G., Deng, X.-W., and Stacey, M. G. (1998) *Gene*, **221**, 35-43.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
23. *Protocol for Ni-NTA Spin Kit* (1994) QIAGEN GmbH, Germany.
24. Topfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H.-H. (1987) *Nucleic Acids Res.*, **15**, 5890.
25. Wood, T. M., and Bhat, K. M. (1988) *Meth. Enzymol.*, **160**, 87-112.
26. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.
27. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
28. Teather, R., and Wood, P. (1982) *Appl. Environ. Microbiol.*, **43**, 777-780.
29. Begiun, P. (1983) *Anal. Biochem.*, **133**, 333-336.
30. Black, G. W., Rixon, J. E., Clarke, J. H., Hazlewood, G. P., Theodorou, M. K., Morris, P., and Gilbert, H. J. (1996) *Biochem. J.*, **319**, 515-520.
31. Zhang, J.-X., and Flint, H. J. (1992) *Mol. Microbiol.*, **6**, 1013-1023.
32. Flint, H. J., Martin, J., McPherson, C. A., Daniel, A. S., and Zhang, J.-X. (1993) *J. Bacteriol.*, **175**, 2943-2951.
33. Olsen, O., Thomsen, K. K., Weber, J., Duus, J. O., Svendsen, I., Wegene, R. C., and von Wettstein, D. (1996) *Biotech.* (N. Y.), **14**, 71-76.