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Stabilization of anti-leukemic enzyme L-asparaginase by immobilization on polysaccharide levan

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Abstract

Biologically active fructose polymer levan from *Zymomonas mobilis* of different molecular mass (75 and 2000 kDa) was covalently coupled to anti-leukemic enzyme *Erwinia carotovora* L-asparaginase. The method used for the immobilization of the enzyme involved periodate oxidation of the polysaccharide, followed by reductive alkylation. A gentle periodate oxidation of levan (oxidation degree $\leq 24\%$) resulted in the highest residual enzyme activity ($\geq 55\%$). The $K_{m(app.)}$ of glycoconjugates was higher than the K_m of native L-asparaginase. The conjugation of L-asparaginase widened the optimum pH range of the enzyme. The electrophoretic mobility in polyacrylamide gel of glycoconjugates obtained was considerably reduced in comparison with native L-asparaginase. Immobilized L-asparaginase showed significantly higher stability in conditions of increased temperature (40°C and 50°C) and prolonged storage (1 month) in aqueous solutions as compared to the native enzyme. The results are discussed in relation to possible explanations of levan-induced enzyme stabilization, as well as to possible applications of immobilized L-asparaginase research.

Keywords: *Erwinia* L-asparaginase; Immobilization; Glycosylation; Levan; Stabilization

1. Introduction

Naturally occurring glycoproteins show unusual stability compared with carbohydrate-free proteins. They have less sensitivity to heat and other denaturing conditions and are more stable in biological medium [1,2]. Chemical methods for the glycosylation of proteins in vitro through glycosidic linkages, such as occurrence in natural glycoproteins, are not available. However, the synthesis of glycoproteins (neoglycoconjugates) involving coupling by linkages of other types is now possible. General neoglycoconjugates coupling chemistry — methods to determine the degree of incorporation — have been well described [1,3–5]. It should be noted that the important advantages of the chemical synthesis of glycoconjugates include enhancements in biological affinities or glycoside "cluster effects" due to the multivalency of attachment in neoglycoconjugates [5]. This may result in the significant stabilization of labile protein molecules. Protein–carbohydrate conjugates have served as model compounds in the study of structure and function of natural glycoproteins [2]. Moreover,

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they can be used to target proteins to a particular cell type for therapeutic purposes. Taken together, neoglycoconjugates have found broad applications in medical, biological, and biochemical research [5].

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1), which hydrolyzes L-asparagine to L-aspartic acid and ammonia, has been extensively used in the treatment of acute lymphoblastic leukemia. Enzymes derived from two bacterial sources (*Escherichia coli* and *Erwinia carotovora*) are in common use [6,7]. The efficiency of the anti-leukemic action of L-asparaginase depends on its various enzymatic properties such as kinetic constants, for example, Michaelis constant (K_m), as optimum pH, stability and the others. The actual problem associated with the therapeutic use of L-asparaginase is its low stability in organism and, accordingly, rapid plasma clearance. One approach to achieve improved function and properties of the enzyme is its chemical modification (immobilization) with various kinds of soluble biocompatible polymers [8–12]. It was reported that polyethylene glycol conjugated L-asparaginase from *E. coli* (pegaspargase) is a safe and effective alternative to native L-asparaginase for clinical anti-leukemia therapy, although pegaspargase should not be routinely substituted for the native enzyme [9]. In the light of the data on glycoproteins mentioned above, it has become apparent that enzymes, which are conjugated with soluble polysaccharides, have a variety of potential applications [2]. But the covalent coupling of enzymes to polymeric materials may result in conformational alterations and significant retention of specific activity. Therefore, many attempts have been made to optimize the chemical glycosylation of enzymes, as well as to obtain evidence regarding the mechanisms involved in carbohydrate-induced stabilization [1,2]. We previously demonstrated that the soluble conjugates of *E. coli* L-asparaginase with dextran and carboxymethyl cellulose showed higher stability against extreme medium conditions, as well as decreased plasma clearance rate in animals as compared to the native enzyme [13,14]. Similar results have been observed for *E. coli* L-asparaginase attached to carboxymethyl chitosan [15] and for *Erwinia* L-asparaginase conjugated with dextran [16,17]. Moreover, in recent experiments, it has been shown that the enzyme bound to clinical dextran has a prolonged plasma half-life in man [16]. These observations led to the suggestion that such glycoconjugates should have therapeutic potential.

The purpose of the present study was to investigate the covalent binding of *Erwinia* L-asparaginase to fructose polysaccharide levan from *Zymomonas mobilis*, which possesses anti-cancer, immunomodulatory, and other biological activity [18,19]. The conjugates obtained from levans with different molecular mass (molmass) and the different degrees of functionalization, were characterized with respect to their enzymatic properties and stability in various medium conditions.

2. Materials and methods

2.1. Materials

E. carotovora L-asparaginase (140 kDa) with specific activity 500 international units (IU)/mg protein was obtained from the Institute of Microbiology and Biotechnology, University of Latvia. Levans from *Z. mobilis 113″S″* (75 and 2000 kDa) were prepared, as previously described [19]. All the other chemicals were purchased from Sigma (St. Louis, MO) or Aldrich (St. Louis, MO).

2.2. Oxidation of levan

Levan with molmass of 75 or 2000 kDa (500 mg) and potassium periodate (0.4–3.0 g) were dissolved in water (300 ml). The mixture obtained was constantly stirred in the dark at room temperature for 1–120 h. Oxidized levan was separated from the unreacted potassium periodate by anion exchange chromatography through the column (1.4×30 cm) loaded with anionite resin Amberlite IRA-400 (Bio Rad, USA) in acetate form. If necessary, purified oxidized levan was lyophilized.

2.3. Preparation of glycosylated Erwinia L-asparaginase (GA)

Sixty milliliters of the oxidized levan (~ 4 mg/ml) were added to 20 ml of L-asparaginase solution in 0.5 M sodium carbonate buffer, pH 9.2.

Molar ratio levan/enzyme in reaction are shown in Table 1. The pH of solution was adjusted to the optimum value (9.0–9.2) during glycosylation by adding small amount of 0.5 M sodium carbonate. The reaction mixture was stirred at room temperature for 3 h. After this time, two-fold molar excess of $NaBH_4$ per aldehyde groups was added and slightly mixed for 1 h at room temperature. The solution of GA with low molmass levan was purified by ultrafiltration on Amicon M-100 membrane passing ~10 equal volumes (~100 ml each) of cold (4–5°C) distilled water. The solution of GA with high molmass levan was extensively dialyzed against cold water. All GA were freeze-dried and kept at 4–5°C until further use.

2.4. Determination of enzyme modification degree

The degree of the modification of amino groups in L-asparaginase was determined by measuring the amount of free amino groups by the method using trinitrobenzene sulfonate [20].

2.5. Assay of L-asparaginase activity

The enzyme activity (hydrolysis of L-asparagine) was determined from the Nessler reagent, as described by Zhagat et al. [21]. L-asparaginase activity was defined as μmol of NH^{4+} formed per minute and expressed in IU.

In the kinetic studies, enzyme activity was determined by phenylhypochloride method, as described by Kaplan [22]. The Michaelis constants (K_m and $K_{m(app)}$) of L-asparaginase and GA were calculated from the Lineweaver–Burk plots of enzyme activity vs. L-asparagine concentrations (10–100 μM) in 0.05 M phosphate buffer, pH 8.0.

2.6. Protein and levan analysis

The protein concentration was determined by the Lowry method with bovine serum albumin as the standard [23]. The levan content was estimated by the resorcinol sulfuric acid method of Roe et al. [24]. The optical density was determined with Shimadzu UV-260 (Japan) spectrophotometer at 540 nm.

2.7. Analysis of levan oxidation degree

The amount of oxidized fructose residues (oxidation degree) in the levan molecule was measured by the iodometric method of Lindenbaum et al. [25], involving the direct determination of aldehyde groups in oxidized levan purified from oxidant ions by anion exchange chromatography, as described above. The oxidation degree (γ) was calculated from the following equation.

$$\gamma = \frac{V_C - V_S}{2 \times 12.4 \times c} \times N_T \times 100\%,$$

where V_C and V_S are the volume of 0.01 N thiosulfate solution in control and sample titration, respectively (ml); c is the levan quantity in sample (g); N_T is normality of thiosulfate solution; 12.4 is the coefficient equal to the amount of aldehyde groups (mmol) under full (100%) oxidation of 1 g levan.

2.8. Gel chromatography

Gel chromatography of the preparations was done on a Sephadex G-200 column (1.2 × 50 cm) equilibrated with the 0.05 M potassium phosphate buffer, pH 8.0. Enzyme samples (500 μl, containing ~500 μg protein) were applied on the column and chro-

Table 1
Effect of molmass of levan and modification degree of L-asparaginase of the enzymatic properties of the conjugates (GA)

Enzyme	Levan		Molar ratio levan/enzyme in reaction	Mass ratio levan/enzyme in the conjugate	Enzyme activity (%)	K_m and $K_{m(app.)}$ $M \times 10^{-5}$
	Molmass (kDa)	Oxidation degree (%)				
Native					100	2.5
GA1	75	24.2	2:1	1.02	90.5	3.6
GA2	75	24.2	5:1	3.22	76.0	5.2
GA3	2000	19.3	1:10	1.50	58.0	9.5

matographed at a flow rate of 18 ml/h at 5°C. Effluent fractions were analyzed by Automated Chromatography System (LKB, Sweden). Uncoupled high molmass levan was detected in effluent fractions by the method of Roe et al. [24].

2.9. Electrophoresis

The enzyme samples were analyzed by disc electrophoresis in 7% polyacrylamide gel as described by Davis [26].

3. Results and discussion

3.1. Glycosylation of Erwinia L-asparaginase

From various methods of enzymes glycosylation, we selected one of the simplest, straightforward, and reproducible method involving the conjugation of *Erwinia* L-asparaginase with oxidized polysaccharide. This method has been successfully used for the immobilization of *E. coli* and *Erwinia* L-asparaginases using oxidized dextran [13,16,17]. Upon the oxidation of levan with potassium periodate, selective scission of chemical bonds between two carbon atoms (C3 and C4) in the fructose ring occurs, forming two reactive aldehyde groups (Fig. 1). The glycoconjugates were prepared by the direct reaction of dialdehyde groups of oxidized levan with ε-amino groups of lysine and N-terminal amino groups of the enzyme. After conjugation, both unstable azomethine linkages, which are formed during the reaction, and unreacted aldehyde groups were simultaneously reduced with sodium borohydride (Fig. 1). Then, the further alkylation of enzyme amino groups was blocked. The oxidation of polysaccharides by periodate is performed in mild aqueous conditions, and leads to easily characterized products with desirable oxidation degree. We optimized oxidation conditions with regard to the amount of periodate (Fig. 2A) and to the oxidation time of levan (Fig. 2B). Moreover, we studied the influence of levan molmass on the level of oxidation using low molmass (75 kDa) and high molmass (2000 kDa) levans (Fig. 2A and B). The maximum oxidation degree of levans (60–70%) was achieved within 5–7 h of oxidation and at molar ratio of potassium periodate per fructose unit of 1.0 in reaction. The longer reaction time (up to and even more than 120 h) or the higher periodate concentrations did not increase the oxidation degree of both low and high molmass levans. In previous experiments, we found that various forms of oxidation products (hemialdals and hemiacetals), which may retard the complete (100%) oxidation of polysaccharide, were not formed (unpublished data). Therefore, the incomplete oxidation of levans may be explained by their high-branched

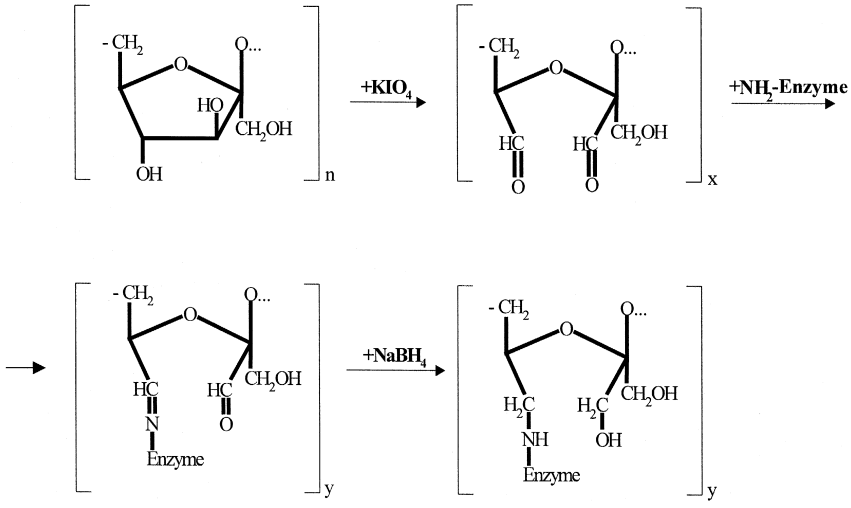

Fig. 1. Reaction scheme of the immobilization of *Erwinia* L-asparaginase.

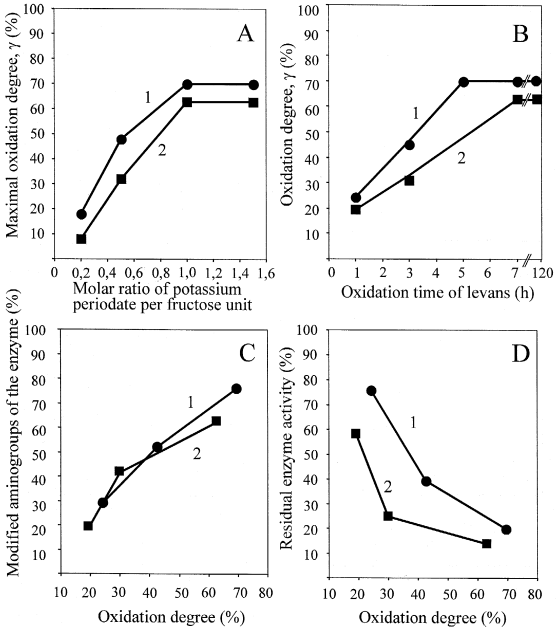

Fig. 2. Effect of various parameters on the oxidation degree of levans (A and B) and structural and enzymatic properties of GA (C and D). (Curves 1 and 2) — conjugates GA2 and GA3, respectively (see Table 1). In (A), oxidation time is 120 h. In (B, C and D), molar ratio of potassium periodate per fructose unit was 1.0.

structures, which prevent the approach of KJO_4 to the molecule inside. Indeed, it was established that bacterial levans with branched morphology exist as compact globular-shaped molecules in aqueous solutions [27,28]. We previously showed that viscosity of levan solutions at low concentrations ($\geq 1\%$) is quite stable and reversible at room temperature over a wide range of pH from 3 to 11 [29]. Therefore, it is reasonable to suggest that degradation of branched oxidized levan during oxidation (pH 3.8–4.0) did not occur. The conjugation of L-asparaginase was performed at pH 9.0–9.2 when the amino groups of the enzyme were non-protonated. The degree of modification of L-asparaginase was assessed by estimating the number of amino groups before and after levan was coupled to the enzyme. Fig. 2C shows that at maximum the oxidation degree of levans (60–70%, oxidation time 5–7 h), more than 60% of the enzyme amino groups were modified. However, this resulted in a significant loss (up to 80–85%) of enzymatic activity (Fig. 2D). On the other hand, a gentle periodate oxidation of levans (oxidation degree $\leq 24\%$) caused the lowest decrease of enzyme activity of GA, and reasonable extent of conjugation of L-asparaginase. Indeed, as we described previously, the best results upon L-asparaginase immobilization on dextran can be obtained for enzyme conjugates with only 15–20% of the amino groups modified [13]. Moreover, it is known that coupling reactions with highly oxidized polysaccharides performed in alkaline medium may result in their degradation [30]. Therefore, we further investigated GA with minimal oxidation degree of levan (19–24%) and modification degree of the enzyme (residual activity > 55%). As shown in Table 1, the loss of L-asparaginase activity due to conjugation was proportional to the molmass of levan, and to the amount of coupled levan (75 kDa). This decrease may be, to a certain degree, explained by the inability of the enzyme and substrate to interact due to increasing steric hindrance by coupled branched levans.

The extent of the coupling reactions and homogeneity of GA were analyzed by gel filtration chromatography on Sephadex G-200 and disc elec-

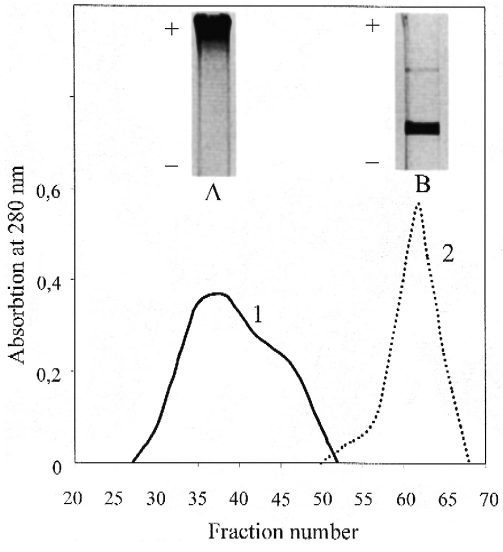

Fig. 3. Homogeneity of immobilized L-asparaginase. (Curves 1 and 2) — elution profiles (Sephadex G-200, column 1.2×50 cm, eluted by 0.05 M phosphate buffer at a flow rate of 18 ml/h, at 5°C) of GA2 and native L-asparaginase, respectively. (A and B) — disc electrophoresis of GA2 and native enzyme, respectively. Samples contained 200 μg of the enzyme. Gels were stained with 0.25% Coomasie blue in 7% acetic acid for 1 h.

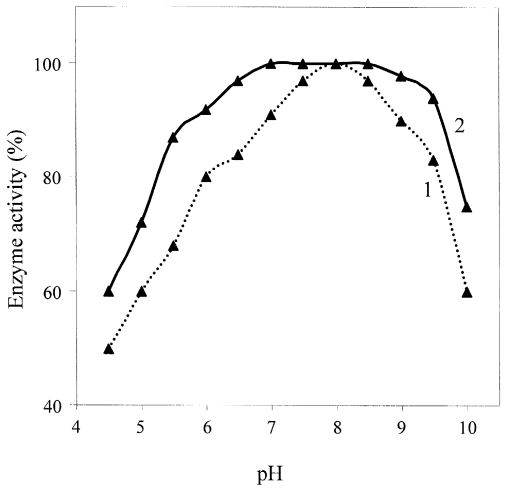

Fig. 4. The pH profile of native L-asparaginase (1) and GA1 (2). A series of buffers was used: sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–8.5) and sodium borate (pH 9.0–10.0).

trophoresis in polyacrylamide gel. The coupling of L-asparaginase to oxidized levans led to the formation of large conjugates, which were eluted at a retention time corresponding to much higher molmass compounds than that of native enzyme (Fig. 3, curves 1 and 2). As seen from electrophoretic analysis (Fig. 3A and B), GA formed a single indistinct band at the top of the gel. This would indicate that the glycosylation of L-asparaginase resulted in greatly decreased electrophoretic mobility of the enzyme. On the other hand, the increased molmass of compounds formed may serve to retard migration into the gel [31]. Our results showed that upon selected coupling conditions, both GA2 (Fig. 3) and other GA obtained (see Table 1) were free of unbound native L-asparaginase. Subsequent experiments were performed using these GA.

3.2. Enzymatic properties of GA

The values of the apparent K_m of GA obtained were 1.5–4 times higher than that of the native enzyme (Table 1). It appears that this increase is not due to a change in L-asparaginase itself, but rather due to diffusional barrier imposed by the branched polysaccharide, especially in the case of high molmass levan (2000 kDa). Upon glycosylation, the optimum pH range of L-asparaginase was significantly broader (Fig. 4). Thus, the pH optimum at 7.0 for L-asparaginase represented 92% of the pH 8.0 optimum, while the optimum at pH 7.0 for GA1 represented 100% of that at pH 8.0.

By incubation of L-asparaginase and three GA at 40°C and 50°C in phosphate buffer for 1 h, we have observed that all GA were more stable to these temperatures than was the native enzyme (Fig. 5). Thus, GA3 retained 95–100% of the initial activity,

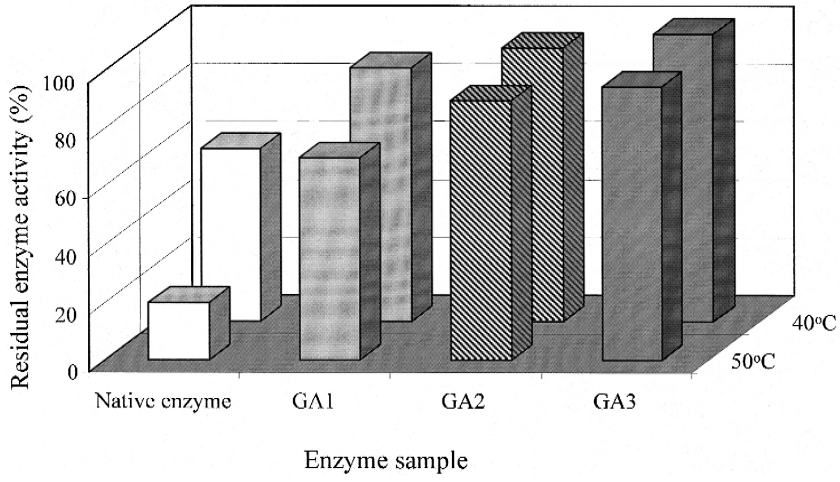

Fig. 5. Thermal stability of native L-asparaginase and GA. The enzyme samples in phosphate buffer, pH 8.0 (1 mg protein/ml) were incubated for 1 h at indicated temperature. Then samples were removed, allowed to cool to room temperature, and analyzed for residual enzyme activity.

whereas native L-asparaginase retained only 20% of its original enzymatic activity at 50°C. The improved stability of GA may be acquired by multipoint attachment of the enzyme molecule to the polysaccharide chain, resulting in greater rigidity of enzyme conformation and in the increase of the activation energy for "uncoiling" the enzyme [32]. Moreover, it was established that native L-asparaginase (four identical subunits) lost the enzymatic activity due to dissociation into inactive subunits by dilution of the enzyme solutions [33]. It seems, therefore, reasonable to suggest that the stabilization of tetrameric L-asparaginase upon glycosylation may be also due to protection from this dissociation, especially in extreme conditions.

We next examined the effect of immobilization on the time stability of GA obtained. After 1 month, all GA retained 90–95% of initial enzyme activity in aqueous solutions (protein concentration 1 mg/ml) at 4°C, as compared to 20% for native L-asparaginase.

In general, the results obtained in this study suggest that the proposed method of glycosylation of *Erwinia* L-asparaginase by its chemical coupling to biologically active fructose polymer levan opens up new possibilities for various practical and theoretical applications of immobilized enzyme (Fig. 6). Undoubtedly, such stabilized conjugates as effective enzyme forms may find application primarily for medical purposes.

4. Conclusions

The anti-leukemic enzyme *Erwinia* L-asparaginase was covalently bonded to biologically active polysaccharide levan from *Z. mobilis* of different molmass (75 and 2000 kDa) by the method of periodate oxidation of levan and following reductive alkylation. The low degree of oxidation of both low and high molmass levans (19–23%) caused the lowest decrease of enzyme activity of glycoconjugates and reasonable extent of the coupling of L-asparaginase to levans. The glycosylation of L-asparaginase under selected experimental conditions increased the values of the apparent K_m, widened the range of pH stability, and reduced the electrophoretic mobility of the enzyme. Immobilized L-asparaginase showed higher thermal (40°C and 50°C) and storage (1 month) stability in aqueous solutions as compared to the native enzyme. We suggest that the immobilization method presented in this paper represents an ap-

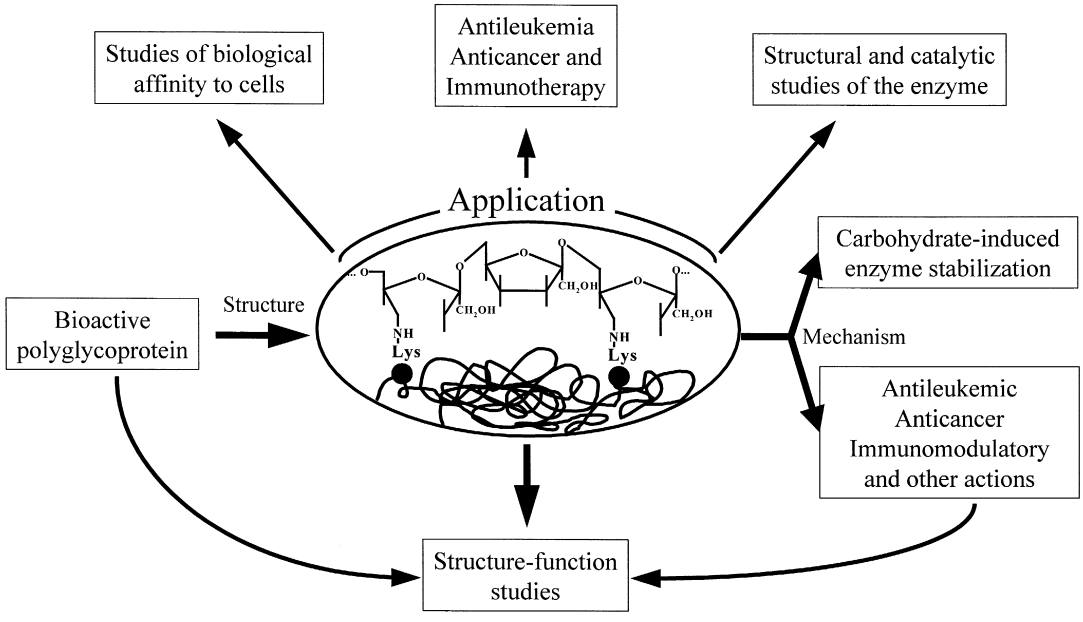

Fig. 6. Possible applications of immobilized L-asparaginase research.

proach to the stabilization of L-asparaginase employed for medical purposes, that may be of general application.

Acknowledgements

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References

[1] J.J. Marshall, M.L. Rabinowitz, Arch. Biochem. Biophys. 167 (1975) 777.
[2] H.K. Blomhoff, T.B. Christensen, Biochem. Biophys. Acta 743 (1983) 401.
[3] Y.C. Lee, R.T. Lee (Eds.), Neoglycoconjugate: Part A. Synthesis, Methods Enzymol. Vol. 242, Academic Press, San Diego, 1994.
[4] Y.C. Lee, R.T. Lee, in: H.-J. Gabius (Ed.), Glycosciences: Status and Perspectives, Chapman & Hall, London, 1997, p. 55.
[5] M.M. Palcic, Glycobiology Catalog, Calbiochem, California, USA, 1998, p. 76.
[6] R.A. Larson, M.H. Fretzin, R.K. Dodge, C.A. Schiffer, Leukemia 12 (1998) 660.
[7] H.J. Muller, J. Boos, Crit. Rev. Oncol./Hematol. 28 (1998) 97.
[8] M.B.F. Martins, A.P.V. Goncalves, M.E.M. Cruz, Bioconjugate Chem. 7 (1996) 430.
[9] L.M. Holle, Ann. Pharmacother. 31 (1997) 616.
[10] A.F. Fernandes, G. Gregoriadis, Biochim. Biophys. Acta 134 (1997) 26.
[11] J. G.Qian, J. Ma, B. Zhou, React. Funct. Polym. 32 (1997) 117.
[12] C. Monfardini, F.M. Veronese, Bioconjugate Chem. 9 (1998) 418.
[13] A.S. Karsakevich, S.N. Shulichenko, M.R. Buka, I.A. Vina, A.P. Kashkin, Khim. Prirodn. Soyed. (Uzbekistan) 6 (1986) 766.
[14] A.S. Karsakevich, O.B. Kinstler, I.A. A.Zh. Dauvarte, I.A. Vina, Vopr. Med. Khim. (Moscow) 34 (3) (1998) 107.
[15] J. G.Qian, J. Zhou, B. Ma, D. He, Sci. China. Ser. B: Chem. 40 (1997) 337.
[16] T.E. Wileman, M. Bennett, J. Lilleymann, J. Pharm. Pharmacol. 35 (1983) 762.
[17] T.E. Wileman, R.L. Foster, P.N.C. Elliot, J. Pharm. Pharmacol. 38 (1986) 264.
[18] V. Liepa, G. Zakenfelds, E. Volpe, Y. Koronova, R. Lapsa, M. Laivenieks, M. Bekers, L. Pospishil, Proc. Latv. Acad. Sci., B 5 (1993) 59.
[19] I. Vina, M. Bekers, A. Karsakevich, R. Linde, S. Gonta, M. Toma, Toma, Proceedings of the International Conference, Biomass for Energy and Industry, Würzburg, Germany, 8–11 June, 1998, p. 460.
[20] A.F.S.A. Habeeb, Anal. Biochem. 14 (1966) 328.
[21] R. Zhagat, I. Shprunka, I. Eiduse, I. Vina, D. Daiya, Proc. Latv. Acad. Sci. 1 (1973) 73, Chemical Series No.
[22] A. Kaplan, Methods Biochem. Anal. 17 (1969) 311.
[23] O.H. Lowry, N.J. Rosenbrough, A.Z. Farr, R.J. Randal, J. Biol. Chem. 193 (1951) 265.
[24] J.H. Roe, J.H. Epstein, N.P. Goldstein, J. Biol. Chem. 178 (1949) 839.
[25] G.M. Lindenbaum, O.A. Mirgorodskaya, B.V. Moskvichov, Khim.-Farm. Zh. (Moscow) 11 (1977) 80.
[26] B.J. Davis, Ann. N.Y. Acad. Sci. 121 (1964) 404.
[27] S.S. Stivala, W.S. Bahary, Carbohydr. Res. 67 (1978) 17.
[28] P.J. Simms, W.J. Boyko, J.R. Edwards, Carbohydr. Res. 208 (1990) 193.
[29] I. Vina, A. Karsakevich, S. Gonta, R. Linde, M. Bekers, Acta Biotechnol. 18 (1998) 164.
[30] R.D. Guthrie, in: M.Z. Wolfrom (Ed.), Advances in Carbohydrate Chemistry Vol. 16, Academic Press, New York, 1961, p. 105.
[31] P.J. Lisi, T. van Es, A. Abuchowski, N.C. Palczuk, F.F. Davis, J. Appl. Biochem. 4 (1982) 19.
[32] D. Shepard, M. Donovan, E. Raghupathy, K.-K. Yeung, A.J. Owen, J.A. Dain, Carbohydr. Res. 118 (1983) 239.
[33] T. Yoshimoto, K. Takahashi, A. Ajima, A. Matsushima, G. Saito, Y. Tamaura, Y. Inada, EBS Lett. 183 (1985) 170.