Preparation and Application of Complexes Based on Biopolymers of Animal Origin

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Abstract. Immobilization of enzymes (IoE) from animal origin on natural carriers increases the system stability; facilitates the separation and accelerates the recovery of the enzyme; makes reuse possible; provides a significant reduction in operating costs. There are numerous IoE methods and systems, including immobilization of various lipases on major carbohydrate biopolymers (chitin, chitosan, cellulose, etc.), discussed in this review. The key points of the most encouraging methods "for increasing the activity and stability" of such biopolymer systems are the "chitosan particle activation" by "ultra-sonication" and multiplicative "addition of glutaraldehyde" to these abovementioned systems. The design of such complex biopolymer preparations (in their various forms) is an important area of modern agrosciences, biomedicine, veterinary, zootechnology and bionanotechnology.

1 Introduction

The preparation and application of functional and sophisticated biopolymer systems (including enzyme complexes) in the numerous forms are the significant and modern areas of bio- and nanotechnologies, human and veterinary medicine, agricultural and food sciences and industries [1-3]. Immobilization of enzymes (IoE) from animal origin on natural carriers increases the system stability; "facilitates the separation and accelerates the recovery of the enzyme"; makes reuse possible; provides a "significant reduction in operating costs" [4-6]. There are numerous IoE methods and systems [7-10] which will be discussed below, including "immobilization of particular triacylglycerolacylhydrolases" (TAGHLs) on the following carbohydrate biopolymers: chitin as functional biopolymer I (FBPI) and chitosan as functional biopolymer II (FBPII) [7-10].

It is important to highlight that TAGHLs (lipases as EC 3.1.1.3) are valuable enzymes catalysing the "hydrolysis" of various acylglycerols ("hydrolytic activity" [11]) and/or special "synthesis" of fatty acid esters ("esterification activity" [12, 13]). TAGHLs can be

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"isolated from various sources" [11], but those from the animal pancreas is the most promising for the technical applications [11-14].

It is well-known that FBPII ("aminopolysaccharide") can be obtained from FBPI ("homopolymer of N-acetylgalactosamine") by deacetylation at particular conditions. Both biopolymers, FBPI and FBPII are considered as "promising renewable polymeric materials for a wide range of applications" [11, 15, 16].

This work is devoted to the comparative description and discussion of the TAGHL immobilization on the carbohydrate biopolymers (FBPI and FBPII) and estimation of the hydrolytic and esterification activity of the obtained systems.

2 Comparative description and discussion

The major TAGHL immobilization methods can be separated by "interfacial adsorption" (adsorption, encapsulation, gel inclusion) and "chemical-binding" (crosslinking and covalent binding) methods [11], as well as their combinations (summarized in the Tables 1 [4-9] and 2 [6-8]).

Table 1. Examples of the TAGHL immobilization on the carbohydrate biopolymers (FBPI and FBPII) and their "hydrolytic activity".

Reference number	Sample	Immobilization, %	Hydrolytic activity
[4]	control	-	3.6 μmole/mg*min.
	beads		3.2 μmole/mg*min.
	FBPII-S*		2.3 μmole/mg*min.
	FBPII-G*		3.0 μmole/mg*min.
[5]	control	-	-
	beads	5-6.5	6.67 unit/g (chitosan)
[6]	control	-	107.4 unit/mg (enz.)
	FBPI	23	14.6 unit/mg (enz.)
	FBPII	91	16.1 unit/mg (enz.)
[8]	flakes	53.7	8.6 μmole/mg*min.
	beads	72.3	42.6 μmole/mg*min.
[9]	A**	33	33.74 unit/g (chitosan)
	B**	74	33.4 unit/g (chitosan)
	C**	63	31.11 unit/g (chitosan)
	D**	74	32.2 unit/g (chitosan)
	E**	86	35.18 unit/g (chitosan)

Notes: TAGHLs – "triacylglycerolacylhydrolases or lipases";
FBPI – chitin as functional biopolymer I ;
FBPII – chitosan as functional biopolymer II ;
GAD – glutaraldehyde;
* FBPII-S — TAGHL adsorbed to FBPII beads ("after sonication" [4]);
* FBPII-G – TAGHL covalently attached to FBPII beads ("after sonication, followed by activation of the surface with GAD" [4]);
** A – TAGHL adsorbed at the "interface" of the FBPII beads [9];
** B –TAGHL chemically bound on FBPII beads [9];
** C – TAGHL adsorbed at the "interface" of the FBPII beads, "followed by binding with GAD" [9];
** D – TAGHL chemically bound on FBPII beads, "washed and then further bound by GAD" [9];
** E – TAGHL chemically bound on FBPII beads, "followed by binding with GAD (including free TAGHL)" [9].

Table 2. Examples of the TAGHL immobilization on the carbohydrate biopolymers (FBPI and FBPII) and their "synthetic activity".

References number	Sample	Immobilization, %	Synthetic activity
[6]	control		$1.23*10^{-3}$ µmole/min.
	FBPI	23	$5.23*10^{-3}$ µmole/min.
	FBPII	91	$2.53*10^{-3}$ µmole/min.
[7]	covalent bonding	32.2 mg/g	0.325 µmole/mg (chitosan)
	without	1.91 mg/g	1750 µmole/mg (chitosan)
	1.3 DAPr[#]	1.51 mg/g	2050 µmole/mg (chitosan)
	1.4 DABu[#]	1.36 mg/g	1950 µmole/mg (chitosan)
	1.5 DAPe[#]	0.67 mg/g	1925 µmole/mg (chitosan)
	1.6 DAHe[#]	0.57 mg/g	1550 µmole/mg (chitosan)
[8]	flakes	53.7	33.3 µmole/mg*min.
	beads	72.3	31.9 µmole/mg*min.

[#] 1,3 DAPr: TAGHL-GAD-1,3-diaminopropane-GAD-FBPII;
[#] 1,4 DABu: TAGHL-GAD-1,4-diaminobutane-GAD-FBPII;
[#] 1,5 DAPe: TAGHL-GAD-1,5-diaminopentane-GAD-FBPII;
[#] 1,6 DAHe: TAGHL-GAD-1,6-diaminohexane-GAD-FBPII.

2.1 Interfacial adsorption

Here only limited major publications [4-9] (summarized in the Tables 1 and 2) concerning combinations of the TAGHL immobilization on the carbohydrate biopolymers (FBPI and FBPII) are going to be discussed.

It is important to highlight that both, "hydrolytic and synthetic activities" of TAGHLs immobilized on FBPII (flakes and porous carriers used) are thoroughly investigated by Pereira Braz and coworkers [8]: 1) "the degree of binding" for TAGHL-FBPII flakes or porous carriers - 54% or 72%, respectively; 2) "the hydrolytic activity" (emulsion olive oil as substrate) - 8.6 or 42.6 μmol/min•mg, respectively; 3) "the synthetic (esterification) activity" - 33.3 or 31.9 μmol/min•mg, respectively [8]. So, for TAGHL-FBPII on porous carriers "almost 5-fold increase in the hydrolytic activity" was obtained, whereas only 4% decrease in "the esterification activity" was found.

In the work of Georgina Bassani and co-workers [1], the "electrostatic interactions" between "positively charged" FBPI or FBPII (75% of deacetylation) and "negative charged" TAGHL are proposed as the main sources of these hybrid formations [11]. The other evidence of the major role of "electrostatic interactions" in the TAGHL-FBPII hybrid formations was shown by their precipitation at a ratio of 1:0.011 (mg/mg). In contrast, there were accurate turbidimetry data showing "not complete dissolution of the precipitate" at ionic strength of 1 mol/L NaCl solution [1]. This allowed the authors [1] to suggest the existence in these hybrids of some "hydrophobic interactions" [11].

There are some works of the Russian colleagues [15, 16] who obtained a 3-component hybrids, i.e. complexes of two enzymes (TAGHL-&-trypsin) on FBPII, with relatively low "hydrolytic activity" (65%) as compared to 90% of those for the 2-component hybrids of TAGHL-FBPII [16]. Previously, these authors in another work [15] "determined that the optimum pH for the immobilization of trypsin was pH 5.8" [15], as well as in the case of the hybrids (TAGHL-&-trypsin on FBPII) [15]. It is important to highlight that "the catalytic activity in the phosphate-citrate buffer is the highest (7.8 U/g)" in the case of TAGHL-&-trypsin on FBPII as compared to the "acetate and succinate buffer systems" [15, 16].

2.2 Covalent binding and hybrid formation

In the work of Matheus Dorneles de Mello and coauthors [4], ultrasonic fragmentation of "low viscosity" FBPII was used in order to increase their "surface area" for better interaction with TAGHL. According to the AFM-images the initial FBPII particles had a diameter of about 2 mm, but after ultrasonic pre-treatment their dimensions were from 50 nm to100 nm. Further treatment of the FBPII surface with GAD gave a particle diameter of 100-200 nm, whereas final TAGHL immobilization the average particle size increased to 1000-2000 nm [4] due to the GAD "cross-linking the biopolymers to each other" [11]. As a substrate, 4-methylumbelliferyl butyrate (MUB) was used. The effectiveness of TAGHL immobilization on various FBPII hybrids: No1) 61% (balls); No2) 64% (balls, sonicated); No3) 84% (balls "treated by ultra-sonication" and GAD) - was evaluated [4]. The high effectiveness of TAGHL immobilization can be explained by the "surface activation" of the FBPII particles by GAD. Finally, the GAD treatment increased "the hydrophobicity of the surface of FBPII particles" that increased the TAGHL sorption at the interface" [11]. It is important to highlight that the "non-ionic surfactant" (such as Triton X-100) addition stabilizes the "active (open) form" of TAGHL and changes its "specific activity and kinetic parameters" [11]. The following data were obtained for TAGHL-FBPII hybrids: No1) 2.2 nmol./min•mg (balls); No2) 2.3 nmol./min•mg (K_m = 1.2 mmol., V_{max} = 1.7 nmol./min.); No3) 3.0 nmol./min•mg (K_m = 1.1 mmol, V_{max} = 4.7 nmol./min.) (balls "treated by ultra-sonication" and GAD), as compared to the activity of the free TAGHL - 3.6 nmol./min•mg (K_m = 0.5 mmol., V_{max} = 2.9 nmol./min.) [4]. These data can be explained by TAGHL immobilization in its "active form" on FBPII "in the presence of Triton X-100" [4]. Additional "thermal stability experiments" (in the range of 25°C-45°C) showed the following "activity increase" for these hybrids: No1) by 1.5 times; No2) by 1.6 times; No3) by 1.8 times as compared to the activity of the free TAGHL. The pronounced activity of these hybrids was at pH optimum of 6.5-7.0.

Another effects concerning the "possibility of re-use" of these hybrids were found: No3) almost "100% activity on repeated use and about 85% after five cycles of use"; No2) only "30% with repeated use with further smooth fall in activity to 10% after 8 cycles" [4]. These results (especially, obtained for No3 TAGHL-FBPII hybrids) are the most promising for biotechnological applications [11].

The most interesting aspects of the work of Desai P.D. and co-authors [5] were connected with the formation and investigation of the TAGHL-GAD-FBPII hybrids. The following parameters of the TAGHL-GAD-FBPII hybrids were determined: $K_m = 3.32 \bullet 10^{-7}$ mol. and $V_{max} = 0.32$ nmol./min. as compared to $K_m = 4.0 \bullet 10^{-7}$ mol. and $V_{max} = 0.32$ nmol./min. for "unbound" TAGHL [5]. These hybrids showed about 50% activity loss after 5 cycles, but "improved heat resistance and storage stability" [5] that could be important for possible applications.

2.3 Advantages of a spacer in the hybrid substrates

The advantages of a diaminoalkane spacer incorporation inside the TAGHL-GAD-FBPII hybrids was shown by Gul Ozyilmaz [7] using their "synthetic activity" in formation of the "isoamyl alcohol ester from isoamyl alcohol and acetic acid" (at the range of 50 mM) as the "substrate to product reaction" [7]. It was found that 1,3-diaminopropane (a one of the 4 different variants studied) was the best spacer for TAGHL-GAD-FBPII hybrids because of their "highest activity" [7]. Another useful approach was shown in this work [7], concerning immobilisation of the prepared TAGHL-FBPII hybrids in the alginate gel and GAD at the optimal concentrations of the components: "TAGHL – about 3.0 mg/mL; sodium alginate - about 1.5%; FBPII - about 1.5%; GAD - about 0.15% and calcium chloride - about 2.0 mol./L, respectively" [7]. In such reactions a "small amount of water is needed for activation" of the TAGHL [7]. The quantitative study showed that during "water content decrease from 80% to 40%" in the gel, the TAGHL activity in these hybrids "gradually increased" [7]. The maximal "synthetic activity" was observed for hybrid-gel preparations "containing 27% moisture", but further decrease in the "water content to 17%" led to the decrease in the hybrid-gel "synthetic activity" [7]. It is important to highlight the opposite directions in the increase or decrease tendencies for the "synthetic or hydrolytic activity" of the TAGHL depending on the polarity of the solvent used: "aqueous media or hexane, heptane, chloroform, toluene, xylene" [1, 7,11]. Moreover, the yield of the isoamyl alcohol ester as final product "increased gradually with time of the reaction" using both the so-called "volume or column method" [7], but at higher rate in the first case, probably due to the higher diffusion and reaction speed in the liquid media. In addition, the "thermal stability study" of these hybrids showed the following results at 40°C: 1) after 5-7 hours the activity of the TAGHL-GAD-FBPII hybrids increased up to 40%, whereas of the TAGHL-GAD-FBPII gels - up to 80%; 2) after 24 hours both these activities were about 90% as compared to the control sample [7]. It is important to highlight the "slow and gradual decrease" in the activity of the both (hybrids and gel) preparations "during storage at 4°C for 60 days" [7].

First, Ali Kilinc and co-authors [6] prepared hybrids of TAGHL with FBPI and FBPII using GAD "which was added before (conjugation) and after (cross-linking) the washing of unbound protein" [6]. Second, the conjugation of TAGHL with FBPII using GAD can be "more appropriate and effective than the crosslinking procedure" [6]. Third, the activity and optimal temperature of the reaction rate for TAGHL-GAD-FBPII hybrids were about 85% at 45°C, but for TAGHL-GAD-FBPI hybrids were about 70% at 35°C as compared to the free TAGHL – "60% in the range from 25°C to 30°C" [6]. It is important to highlight that the "immobilization efficiency in phosphate buffer (pH 7.0)" for TAGHL-FBPI hybrids was low (23%), whereas for TAGHL-FBPII hybrids was high (91%), but all hybrids showed relatively high stability and activity even "upon repeated use": for TAGHL-FBPI or TAGHL-FBPII

hybrids were 88% or 67%, respectively, of their "original activity of tributyrin hydrolysis after repeated use at least 10 times" [6]. In addition, the remainder activity for TAGHL-FBPI or TAGHL-FBPII hybrids were 86.5% or 67%, respectively, of their initial hydrolytic activity after their storage "in wet form at 4°C for about 45 days, whereas the free enzyme retained only 20% of its activity" [6]. Thus, the so-called "semi-inactivation period" [6] for both, TAGHL-FBPI and TAGHL-FBPII hybrids were in the range of 11-14 hours.

On the other hand, both, TAGHL-FBPI and TAGHL-FBPII hybrids were effective in "catalysing the esterification reaction of fatty acids and fatty alcohols" with medium carbon chains {caprylic (C8) and lauric (C12) fatty acids} and long carbon chain {palmitic (C16) fatty acid}, i.e. their "esterification activity" determined by the "high performance liquid chromatography" was 2-4 times higher "than for the free enzyme, respectively" [6]. The maximal "esterification activity" of TAGHL-FBPI or TAGHL- FBPII hybrids was found only using a pair of substrates: "lauric acid (C12) and fatty alcohols (n-butanol or n-octanol)" [6], whereas for free TAGHL only the "esterification reaction of lauric acid with n-octanol was observed" [6]. It can be explained by some steric-problems in the formation of the particular "acyl-enzyme complex which is then reacted with an alcohol to produce an ester" in the cases of the fatty acids with long carbon chains (higher than C12) and medium carbon chains (lower than C12) [6, 11].

3 Conclusions

The results discussed above showed that the most promising systems are TAGHL-GAD-FBPI or TAGHL-GAD-FBPII "pre-treated with ultrasound (to increase the particle surface area)". The high effectiveness and stability of TAGHL immobilization can be explained by the "surface activation" of the FBPII particles by GAD. The advantages of a "diaminoalkane spacer" incorporation inside the TAGHL-GAD-FBPII hybrids (the best spacer-form is 1,3-diaminopropane) was shown. To our opinion, the conjugation of TAGHL with FBPII using GAD can be "more appropriate and effective than the crosslinking procedure". It is important to highlight that both, TAGHL-FBPII and TAGHL-FBPI hybrids, show balance between the "hydrolytic and synthetic activities" depending on the definite conditions.

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