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# Immobilized Alcalase on Micron- and Submicron-Sized Alginate Beads as a Potential Biocatalyst for Hydrolysis of Food Proteins

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**Abstract:** Enzymatic hydrolysis of food proteins is convenient method to improve their functional properties and physiological activity. Herein, the successful covalent attachment of alcalase on alginate micron and submicron beads using the carbodiimide based chemistry reaction and the subsequent application of the beads for egg white and soy proteins hydrolysis were studied. In addition to the electrostatic extrusion technique (EE) previously used by others, the potential utilization of a novel ultrasonic spray atomization technique without drying (UA) and with drying (UAD) for alginate submicron beads production has been attempted. The immobilization parameters were optimized on microbeads obtained by EE technique (803 ± 23 μm) with respect to enzyme loading and alcalase activity. UA and UAD techniques resulted in much smaller particles (607 ± 103 nm and 394 ± 51 nm in diameter, respectively), enabling even higher enzyme loading of 671.6 ± 4 mg $g^{-1}$ on the carrier and the highest immobilized alcalase activity of 2716.1 IU $g^{-1}$ in the standard reaction. The UAD biocatalyst exhibited also better performances in the real food system based on egg white or soy proteins. It has been shown that the immobilized alcalase can be reused in seven successive soy protein hydrolysis cycles with a little decrease in the activity.

**Keywords:** alcalase; protein hydrolysis; alginate submicron beads; covalent immobilization; electrostatic extrusion; ultrasonic spray atomization technique



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## 1. Introduction

Alcalase (EC 3.4.21.14), is a commercial crude protease produced from *Bacillus licheniformis* which belongs to the class of a non-specific serine-type endoprotease. It is used extensively in food industry with special concern to produce protein hydrolysates with better nutritional or functional properties than intact proteins [1]. The peculiar properties of bioactive peptides as a functional food source have led to much interest in the isolation of peptide fractions or individual peptides from enzymatic hydrolysates of different plant and animal proteins. However, much of this work has involved the use of free enzyme in a batch process [2–4]. For the development of a commercial process for the production of protein hydrolysates, it would be more cost effective to use an immobilized enzyme for protein hydrolysis, since the immobilized biocatalyst can be recovered at the end of a

hydrolysis cycle and reused. Additional advantages include high productivity, automation, continuous processing, easy product recovery and precise control of the extent of reaction, excluding the requirement of post-hydrolysis heat processing for enzymatic inactivation and the possible protein or peptide structure modification caused by this treatment [5–7].

A selection of both appropriate carrier and immobilization method is very important in order to improve enzyme stability and in some cases even activity [8,9]. Alginate is a promising carrier since it is a natural highly negatively charged polysaccharide synthesized by brown seaweeds and by soil bacteria, that forms rigid gel-like structures in presence of divalent cations, usually $Ca^{2+}$ [10,11]. Various methods of alginate gelation (external, internal and cold gelling) have been developed so far, as well as methods for obtaining calcium-alginate particles (extrusion, emulsion, spray-drying, complex coacervation) [12–14]. Most of the existing methods refer to production of particles in the size range 100–2000 μm, while only scarce literature can be found on submicron alginate beads. For the enzyme immobilization purposes, submicron alginate beads (<1 μm) have a number of advantages over larger beads providing a solution to the generally contradictory issues regarding the optimization of immobilization system like much greater mechanical strength, minimum diffusion restriction, maximum surface area per unit mass, and high enzyme loading [15]. Furthermore, they can easily flow through narrow sprays and channels that would be blocked by larger beads.

Recent advances in nanotechnology have delivered diverse nanostructured materials that are more effective for enzyme immobilization like mesoporous silica nanoparticles or metal ions modified amino-functionalized hollow mesoporous silica spheres (HMSS-$NH_2$-Metal), providing high surface areas, ordered mesopores and abundant modifiable site on surface [16,17]. Alcalase immobilized on HMSS-$NH_2$-$Fe^{3+}$ has shown to be very interesting biocatalyst and offered advantages in terms of amidase and proteolytic activity and stability over their free form, very likely due to the favorable coordinate covalent binding of the enzyme to the carrier [17]. However, most of the methods described in literature for production of alginate nanobeads, nanoaggregates and nanocapsules are complicated to perform, often involving the use of various chemicals (besides alginate and a $Ca^{2+}$ ion source) and have a limited ability to control particle size (aggregate generation). In addition, most of them are not suitable for obtaining large amounts of beads, which means that they are not of major importance for industrial application [18–21]. In this paper, two types of calcium alginate beads were synthesized: microbeads and submicron beads. The alginate submicron beads were obtained by a custom-made system, which employs spraying the sodium alginate solution using an ultrasonic spray atomization unit. Subsequently, the alginate aerosol is dried and then carried by air stream to a column with wetted walls, where the gelation with calcium chloride solution takes place. Such a system has two novelties in comparison to conventional ultrasonic spray atomization. One is drying of the beads, which enables generation of the functional beads from diluted alginate solutions. Namely, the drying of the alginate matrix leads to the increase of the alginate concentration and the particles solidity. Another novelty is the way of collecting beads using a column with wetted walls that overcomes many problems including column clogging and formation of agglomerates at column inlet and outlet that occur when alginate is not in aerosol form. The other type of alginate beads, microbeads, was obtained by electrostatic extrusion method which is based on the process of alginate droplet formation under the influence of electrostatic forces [10,13].

The most popular method for immobilization of various enzymes using alginate beads is entrapment in a matrix. [10,11,22,23]. However, for alcalase immobilization, several aspects need to be considered such as the unique features of the substrates, usually large macromolecules, causing internal diffusion limitations of this large molecule within the catalyst and the enzyme tendency towards autolysis. Furthermore, enzyme immobilization by entrapment in polymer matrix is accompanied by a decrease in catalytic efficiency in successive hydrolytic cycles, as a consequence of the enzyme leakage. On the other hand, covalent binding of various enzymes to calcium alginate beads using carbodiimide chem-

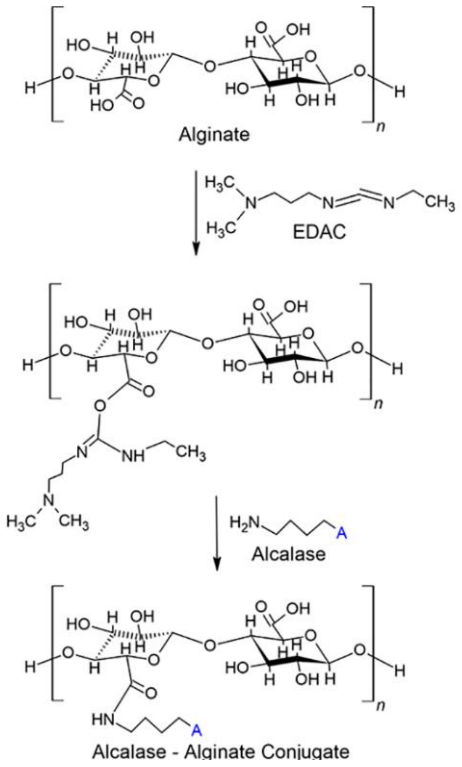
istry has been described as a suitable method to achieve stabilization of several enzymes via multipoint covalent attachment like β-galactosidase [24], acetylcholinesterase [25], α-amylase [26] and others. Although enzymes covalently attached to alginate beads offer some particularly attractive advantages for many biological applications, to the best of our knowledge, there are no studies reported in the literature regarding covalent immobilization of alcalase on alginate micro- or submicro-beads. Generally, the covalent immobilization of proteases and their application to catalyze hydrolytic reactions in real food systems have been investigated to a far lesser extent than applications including free enzymes [27].

In this paper, we examined the feasibility of alcalase covalent immobilization on both calcium alginate microbeads and submicrobeads, activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). The immobilization conditions (enzyme concentration, buffer pH and molarity, EDAC/carrier mass ratio, time of EDAC treatment and immobilization time) were optimized. The yields of immobilization, enzyme binding capacities and activity of the immobilized enzyme were determined for micron-sized and submicron-sized alginate beads and the results were compared. The optimally produced biocatalysts were then used in the industrially feasible reactions including hydrolysis of egg white and soy proteins.

## 2. Results and Discussion

### *2.1. Covalent Immobilization of Alcalase*

The covalent immobilization of alcalase onto alginate microbeads was carried out by amide bond formation using carbodiimide as a coupling agent. EDAC reacts first with the carboxyl groups of alginate beads and forms an O-acylisourea intermediate. This intermediate reacts promptly with an amino group of the enzyme to form an amide bond and releases an isourea by-product. The schematic illustration of the alginate-alcalase conjugation through amide bond between the amino group of enzyme and carboxyl group of alginate mediated by EDAC is presented (Scheme 1).




**Scheme 1.** Possible mechanism of alginate beads activation by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and alcalase covalent immobilization to EDAC-activated alginate beads.

### 2.2. Beads Characterization

The mean volume diameter of alginate microbeads produced by electrostatic extrusion with immobilized enzyme was 803 ± 23 µm with the span of size distribution equal to 1.3 ± 0.1. The average size of alginate submicron-sized beads produced by ultrasonic spray atomization (UA) with immobilized alcalase reached 0.607 ± 0.103 µm with polydispersity index of 0.463 ± 0.053, while their immobilized dry counterparts (UAD) were even smaller, 0.394 ± 0.051 µm. zeta potential (ZP), which reflects the electric charge on the particle surface was not significantly different between beads variations, and ranged from −6.2 mV ± 5.3% for blank alginate beads, and −28.9 mV ± 4.4% for immobilized beads, which is close to the limit of the physical stability of colloidal systems (−30 mV). However, it appeared that the enzyme coating caused decrease in the zeta potential as compared to the blank alginate beads, revealing that the enzyme layer significantly prevent the aggregation and adhesion of the beads. This decrease in zeta value may be attributed to the fact that most of the $NH_2$ groups on the surface of enzyme molecules were conjugated with activated alginate beads, allowing the negatively charged amino acid residues to remain free and to predominate. The results suggested that by drying the aerosol with the help of a heater connected to the apparatus for ultrasonic spray atomization, submicron-sized particles were obtained. Both, micron and submicron beads increased in sized upon enzyme immobilization as their blank counterparts obtained by extrusion technique (EE), UA and UAD were 698 ± 0.21 µm, 428 ± 78 nm, and 261 ± 33 nm, respectively (Table 1).

**Table 1.** Beads diameter and zeta potential.

| Title 1 | Title 2 | Zeta Potential (ZP) |
|---|---|---|
| EE beads | 698 ± 0.21 | −6.34 ± 0.33 mV |
| UA beads | 0.428 ± 0.078 | −6.15 ± 0.26 mV |
| UAD beads | 0.261 ± 0.033 | −6.17 ± 0.27 mV |
| alcalase immobilized on EE beads | 803 ± 23 | −28.6 ± 1.40 mV |
| alcalase immobilized on UA beads | 0.607 ± 0.103 | −27.8 ± 1.69 mV |
| alcalase immobilized on UAD beads | 0.394 ± 0.051 | −30.3 ± 1.11 mV |

EE beads: alginate microbeads produced by electrostatic extrusion; UA and UAD beads: alginate submicron-sized beads produced by ultrasonic spray atomization without drying and with drying, respectively.

The SEM images of blank alginate beads and activated alginate beads with immobilized alcalase produced by EE are presented in Figure 1. The EE beads appeared as round particles (Figure 1a,b) with different surface morphology. Namely, after drying of EE alginate microbeads directly in the SEM chamber without special protection, shrinkage of the beads occurred far more severe in case of blank (Figure 1a) compared to the beads with immobilized enzyme (Figure 1b). In the high magnification views of the respective surfaces, the microscale roughness was observed especially notable in case of the blank beads while surface of the beads with immobilized enzyme appeared as a more compact (Figure 1d) compared to enzyme-free beads (Figure 1c). It seemed that exposing to drying exerted smaller effects on alcalase-immobilized alginate microbeads than on blank microbeads probably as the result of a lower number of hydrophilic groups on enzyme-immobilized microbeads, since covalent immobilization of alcalase occurred via carboxyl groups of alginate. Similar to our observation, more compact surface of tannase-loaded calcium alginate beads versus empty alginate beads was already reported, and the authors hypothesized that tannase hydroxyl groups formed intermolecular hydrogen bonds with alginate carboxylic groups [28].

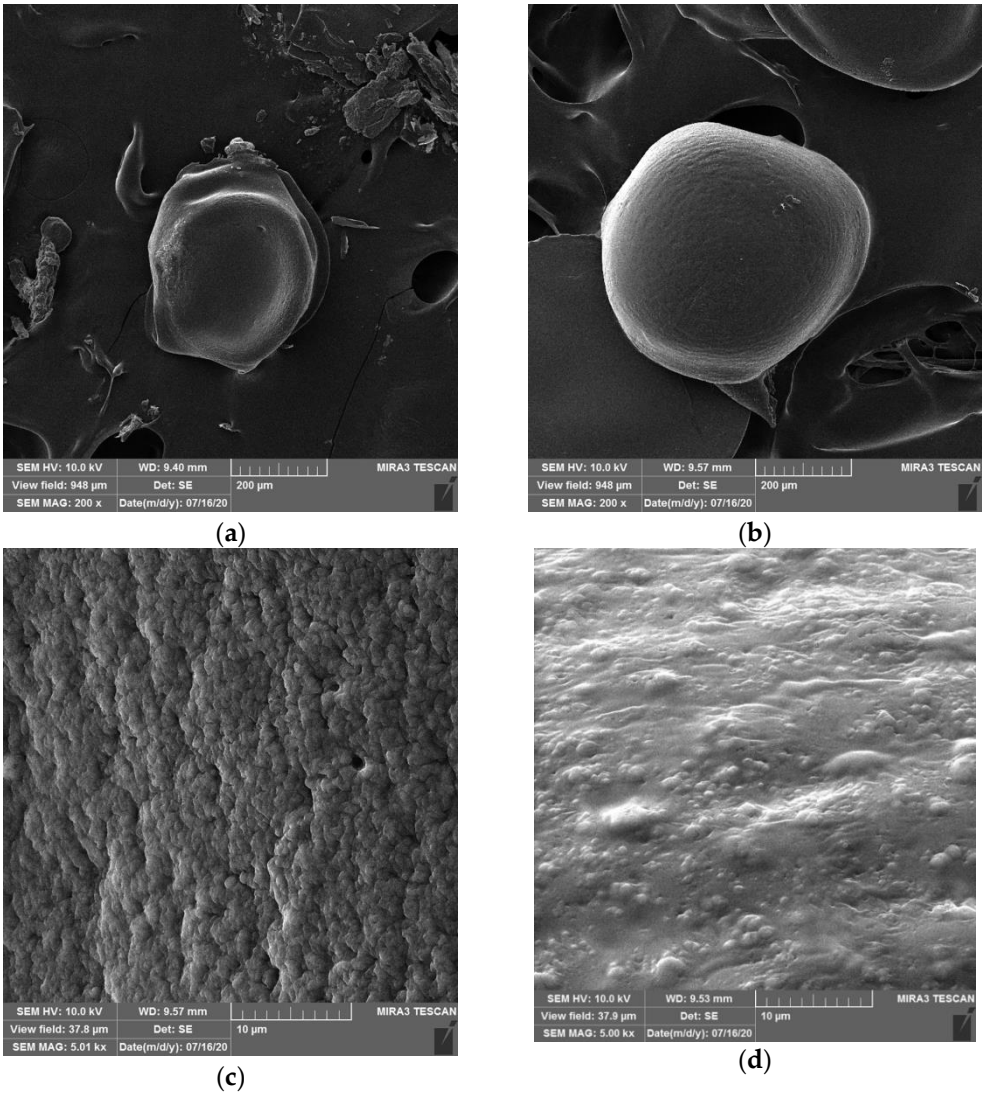



**Figure 1.** SEM micrographs of (**a**) blank alginate EE microbeads at 200× magnification; (**b**) activated alginate EE microbeads with immobilized alcalase at 200× magnification; (**c**) blank alginate EE microbeads at 5000× magnification; (**d**) activated alginate EE microbeads with immobilized alcalase at 5000× magnification.

### *2.3. FT-IR Analysis*

The stretching vibrations of -OH bonds of pure alginate appeared in the wavenumber range from 3000 to 3700 $cm^{-1}$, while stretching vibrations of aliphatic -CH are observed at 2925 $cm^{-1}$ (Figure 2). The bands around 1632 $cm^{-1}$ and at 1385 $cm^{-1}$ can be attributed to asymmetric and symmetric stretching vibrations of the carboxyl ion, respectively. The bands at around 1107 and 935 $cm^{-1}$ can be attributed to the C-O stretching vibration of the pyranosyl ring and the C-O stretching with contributions from C-C-H and C-O-H deformations. The band at 1033 $cm^{-1}$ corresponding to CO stretching is also characteristic of alginate. Additionally, bands appearing around 885 and 815 $cm^{-1}$ can be assigned to mannuronic and guluronic acids, respectively, which are both present in the alginate structure. Several distinctions can be noticed in spectra of alginate-EDAC and alginate-alcalase compared with alginate. The increase in all peak intensity in both spectra (alginate-EDAC and alginate-alcalase conjugates) appeared to be due to activation of bead with EDAC. The original hydroxyl peak (3447 $cm^{-1}$) became a broader peak and moved to the lower frequency (3413 $cm^{-1}$), which should be due to the superposition of the stretching vibration of $\alpha$-$NH_2$ on alcalase. Furthermore, the peaks at 1632 $cm^{-1}$ and 1385 $cm^{-1}$ (both

characteristic for carboxyl ion) in spectrum of native blank beads moved to 1619 $cm^{-1}$ and 1416 $cm^{-1}$, respectively in spectrum of alginate-alcalase and increased in intensity; it was resulted by the overlap between the stretching vibration of α−COOH and the variable angle vibration of α-$NH_2$. Additionally, new peaks appeared, at 1300 $cm^{-1}$ and 1040 $cm^{-1}$, which were in the region characteristic to N-H or C-N bonds. Namely, both EDAC and amino acid residues attached to the polymer backbone may produce new nitrogen–hydrogen or nitrogen–carbon bonds. Similarly, Davidovich-Pinhas et al. have observed new peaks located in the range of 1240–1700 $cm^{-1}$ in alginate-cysteine conjugates after activation of alginate by EDAC agent [29]. An extra peak was also detected in spectrum of alginate-alcalase at 928 $cm^{-1}$ which is in the region of polyaromatic hydrocarbons.

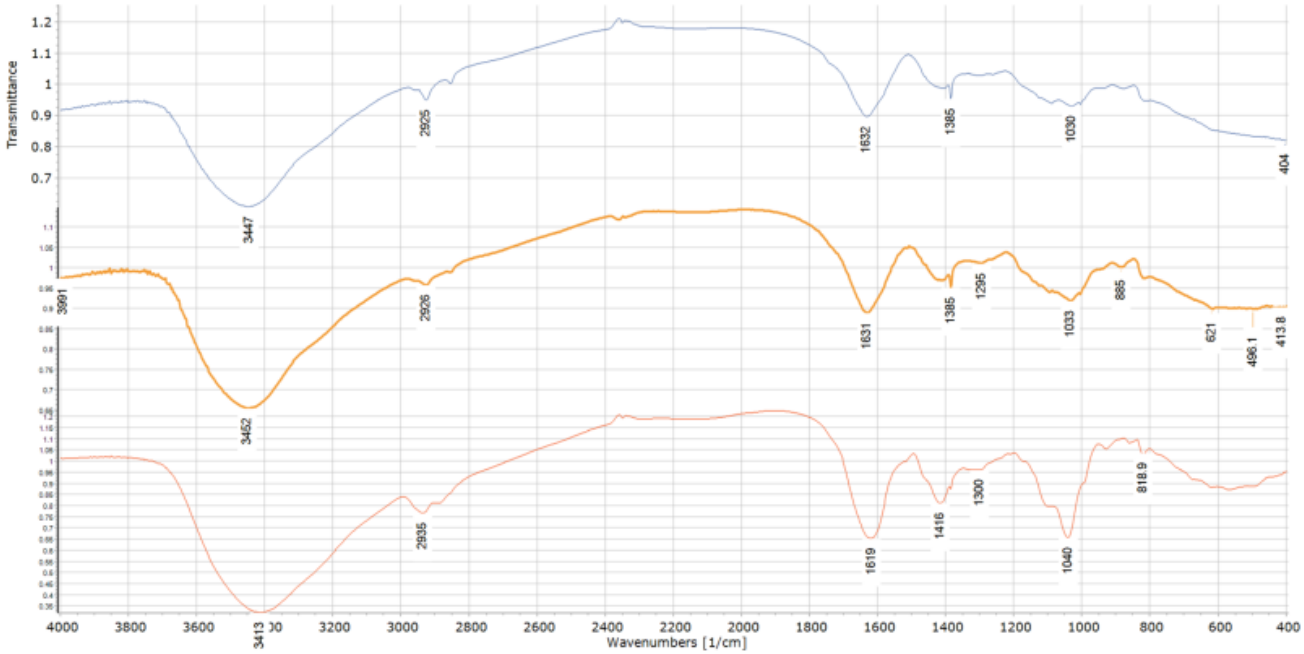



**Figure 2.** FT-IR spectra of investigated sample: (-) pure alginate beads (**blue line**); (-) activated alginate beads (**orange thick line**); and (-) immobilized alcalase-alginate beads (**orange thin line**).

### *2.4. Optimum Conditions for Alcalase Immobilization*

Optimal immobilization conditions are crucial for multipoint covalent attachment and enzyme-carrier reaction [9]. Therefore, the effects of varying initial alcalase/carrier mass ratio of the coupling solution (2–33.6 mg alcalase/g microbeads) on the total protein loading and enzyme coupling yield of the alginate microbeads obtained by electrostatic extrusion (EE) were investigated. The results are shown in Figure 3.

The increase of the initial enzyme/carrier ratio resulted in a linear increase of the enzyme loading, reaching the maximum amount of the alcalase bound of 592.3 ± 6.7 mg $g^{-1}$ dry carrier. The enzyme-coupling yield was influenced by the initial enzyme concentration at a less extent, so that it leveled off at about 97.1% with the initial enzyme/carrier ratio up from 8.4 mg $g^{-1}$. Other publications on alcalase immobilization have shown lower or comparable values for enzyme loading with the coupling yield in the wide range of values. Thus, Pessato et al. attained 0.028 mg protein $g^{-1}$ carrier with glyoxyl-agarose beads [30]. Corîci et al. physically entrapped alcalase in glass sol–gel matrices using alkoxysilanes of different types mixed with tetramethoxysilane at different ratios; the authors achieved entrapped protein content between 83 and 163 mg $g^{-1}$ gel depending on the matrix formulation [31]. Ferreira et al. covalently immobilized alcalase onto silica derivatives of different pore size and surface chemistry (terminal amino versus hydroxyl groups) and reached the loading of 2.4–6.3 mg of protein per gram of silica with a percentage of

immobilized protein 27–70% [32] while Zeng et al. for alcalase immobilized on HMSS-$NH_2$-$Fe^{3+}$ achieved excellent enzyme loading of 227.8 ± 23.7 mg $g^{-1}$ [17]. In a recent study, alcalase was immobilized by various mechanisms (adsorption, enzyme crosslinking and covalent binding) to activated chitosan microbeads; the authors reported the enzyme loading in the range of 116 to 340.2 mg $g^{-1}$, with the enzyme coupling yield of 96.4 to 98.7%, depending on the method used for obtaining chitosan microbeads (electrostatic extrusion versus inverse emulsion techniques) and the matrix formulation (chitosan/cross-linker ratio) [33]. A high enzyme loading achieved herein can be explained by non-selective coupling of the enzyme to the matrix causing the formation of multiple attachment of active enzyme's functional groups with the alginate matrix.

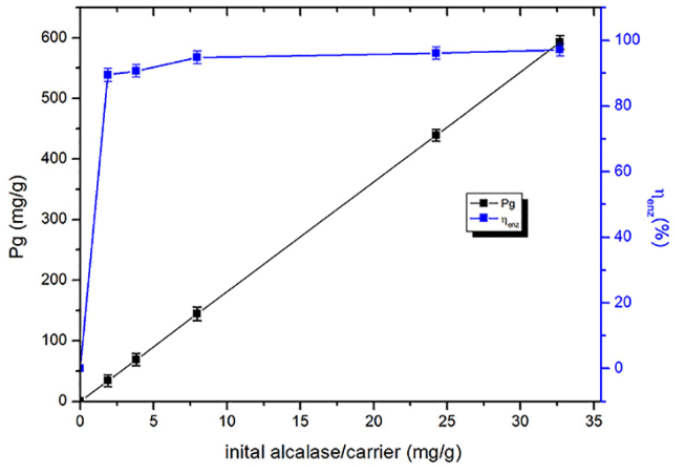



**Figure 3.** Effects of the initial alcalase/carrier ratio on the protein loading ($P_g$) and enzyme coupling yield ($\eta_{enz}$).

We studied the effects of mass of the biocatalysts obtained by varying immobilization parameters such as initial enzyme concentration, buffer pH, buffer molarity, time of EDAC treatment, EDAC/microbeads mass ratio, and time of immobilization on specific activity (activity per dry weight of beads) of the alcalase-alginate EE biocatalyst. The results are shown in the Figure 4. In general, the biocatalyst activity increased with the mass of immobilized enzyme used in the process up to some level, and then reached a plateau. The activity of the immobilized alcalase increased with the enzyme concentration in the initial coupling solution. It appeared that the highest number of enzyme units in the coupling solution of 5.32 IU provided the highest activity of the immobilized system (Figure 4a) and it was used in further studies. In order to avoid dissolution of calcium alginate, alginate-EDAC reaction was carried out in Tris-HCl buffer although phosphate buffer has been used in most previously published studies [34,35]. The major disadvantage of calcium alginate is sensitivity to calcium chelators such as phosphate and lactate. Concerning the effect of buffer pH, the immobilized enzyme obtained with the Tris-HCl buffer of pH 7 showed the lowest activity, and the activity of the biocatalyst increased with the buffer pH up to pH 8.5 and then it started to decrease with further increase (Figure 4b). Namely, the repulsion among the carboxyl groups of alginate was more intensive in a solution with a higher pH value, which resulted in more severe enlargement of the lattice size of the networks so that functional carboxyl groups became more available for conjugation with EDAC. However, beyond critical values, the whole crosslinking network started to disassemble. The increase of the molarity of Tris-HCl buffer led to decrease of the activity of the immobilized enzyme, so that the highest activity showed the biocatalyst obtained with 50 mM Tris-HCl buffer of pH 8.5 (Figure 4). This effect can be associated with pI-dependent swelling behavior of calcium alginate, since the equilibrium swelling of alginate calcium gels decreased with an increase in buffer ionic strength [36].

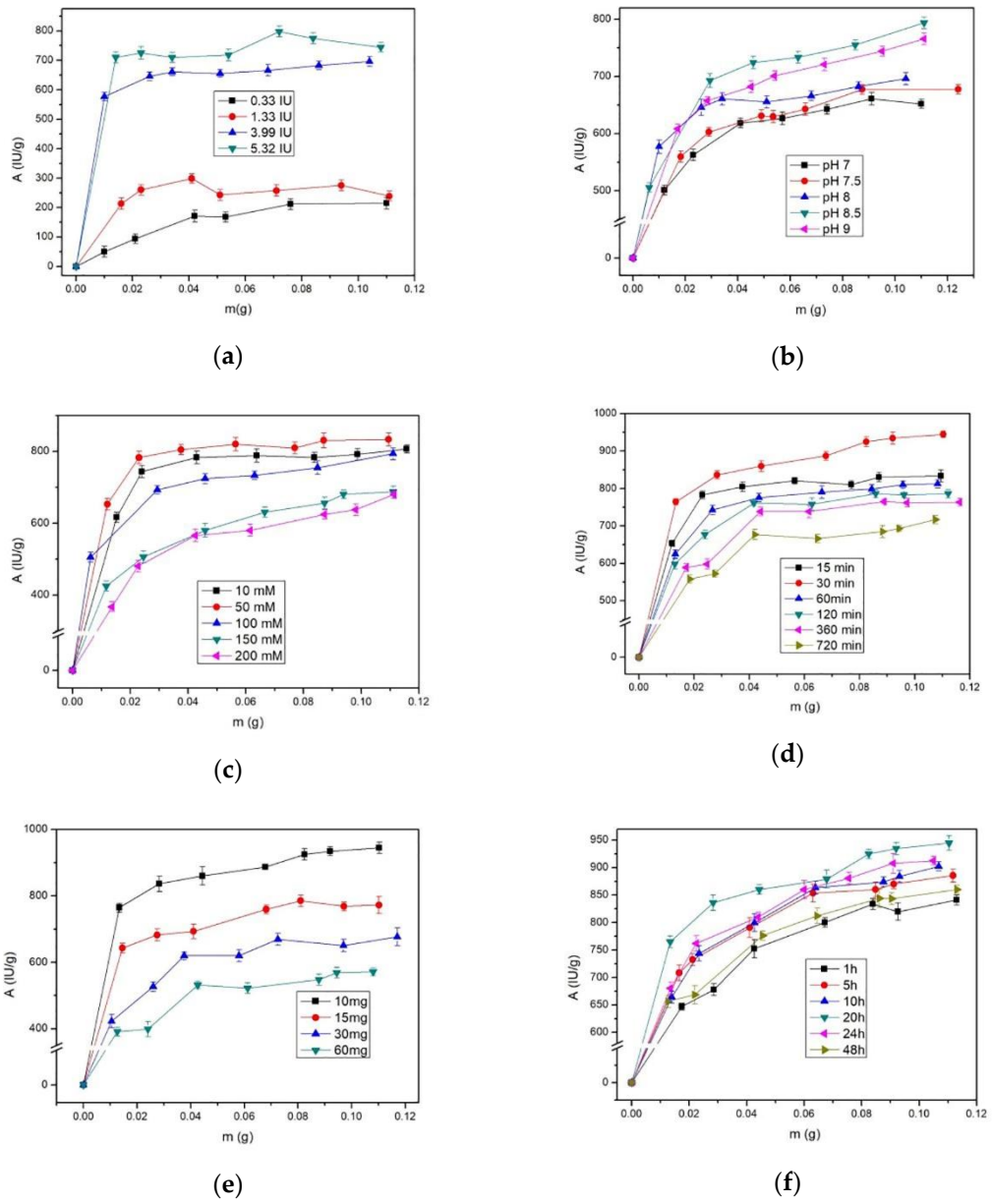



**Figure 4.** Effects of mass of the alcalase-alginate biocatalyst (immobilized alcalase) obtained by varying (**a**) enzyme concentration; (**b**) buffer pH; (**c**) buffer molarity; (**d**) time of EDAC treatment; (**e**) initial EDAC/microbeads mass ratio; and (**f**) time of immobilization on activity of the alcalase-alginate biocatalyst obtained from alginate microbeads produced by EE.

Time of reaction with EDAC (30–720 min) was also studied (Figure 4d). It can be concluded that 15 min was not enough for the activation process to complete but reaction time longer than 30 min resulted with reduced enzyme activity. This can be explained by apparently an excessive bead crosslinking by EDAC, resulting in subsequent inter-crosslinking among the enzyme molecules, which in turn may cause enzyme deactivation. Therefore, 30 min was selected as optimal activation time.

The effect of the EDAC/carrier mass ratio (10–60 mg/0.5 g) used for the microbead activation on activity of the immobilized enzyme is presented in Figure 4e. The highest activity was achieved with 10 mg of EDAC per 0.5 g of beads, and decreased with increase of the EDAC mass. Several reasons could account for this result. Namely, EDAC has functional groups that can react with carboxyl groups of the calcium alginate microbeads (as presented in Scheme 1). However, carboxyl groups of enzyme amino acids may also react with EDAC, as more intensively as EDAC concentration was increased. Namely, the

active site of alcalase includes common catalytic triad made up of three amino acids, serine, aspartate and histidine. It appeared that the carboxyl group of aspartates could react with an excess of EDAC resulting to reduced enzyme activity upon immobilization. Another possible explanation is that the multipoint attachment of enzyme caused by introducing a large number of functional groups in the carrier as well as the steric hindrance caused by overloading of the enzyme also led to the inaccessibility of enzyme to substrate. The problem of reactivity of enzyme active sites with amino group of EDAC was also noticed by Tee et al. who investigated immobilization of α-amylase by covalent binding to alginate matrix; the authors proposed a two-step conjugation protocol for protection of the enzyme active site: after reaction of the calcium alginate beads with EDAC, further reactivity of EDAC with carboxyl groups was quenched by addition of 2-mercaptoethanol to the reaction medium which prevented EDAC from reacting with the active site carboxyl groups of the enzyme during the protein immobilization step [26]. With the aim to gaining better knowledge on how an excess of EDAC affected the activity of the immobilized alcalase, we added 2-mercaptoethanol in the second step and repeated the experiment. The determined biocatalyst activities revealed no significant differences among the various probe tested (Supplementary Figure S1), showing that the contribution of the first cause to the deactivation of enzyme was negligible. Multiple enzyme attachment to the carrier, which limited the flexibility of the polypeptide chain or overloading of the enzyme, seemed to account for the reduced enzyme activity by using higher EDAC concentration.

The incubation time of immobilization process (15–720 min) also expressed effect on alcalase activity (Figure 4f). It seemed that if exposure time was less than 20 h, there were still available carboxyl groups of the alginate matrix for binding, while with a prolongation of contact time above 20 h, the enzyme deactivation was progressing with time. Henceforth, the optimal immobilization conditions were the following: 5.32 IU of alcalase, 50 mM Tris-HCl buffer pH 8.5, 30 min of activation with 10 mg of EDAC per 0.5 g of beads, 20 h immobilization time.

The other type of biocatalysts was also made from alginate particles, which were synthesized from alginate solutions of 0.4 and 0.5% (*w*/*v*) concentrations by ultrasonic spray atomization technique without drying (UA) and with drying (UAD) of the aerosol generated by ultrasonic atomizer. The obtained UAD and UA submicron beads were used for the alcalase immobilization under previously determined optimal conditions. In fact, thus obtained UAD and UA biocatalysts are considerably smaller than most of the enzyme-alginate beads reported in literature [37,38]. Both biocatalysts had high binding capacities since coupling yield of 94 to 97% was achieved, respectively. The activity of the immobilized alcalase increased with its mass in the reaction, as expected (Figure 5).

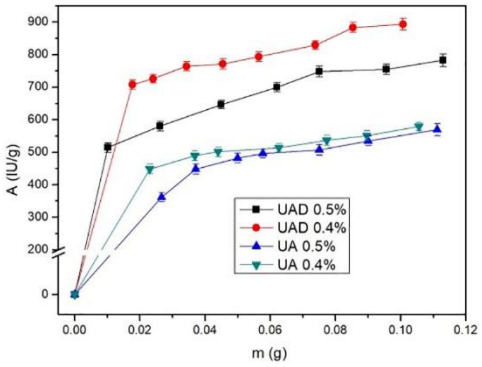



**Figure 5.** Effect of the biocatalysts mass on the activities of alcalase immobilized onto alginate submicron beads produced by ultrasonic atomization.

Furthermore, the UAD biocatalyst expressed higher activity in comparison to its' UA counterpart. This can be explained by two facts: (1) the number of reactive groups per mass unit of alginate particles increased upon drying, and; (2) UAD particles were far

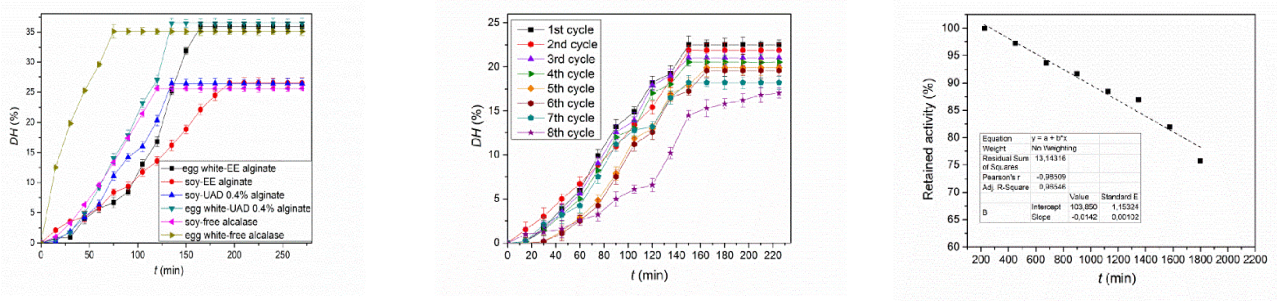
smaller than UA, thus having higher specific surface area. In addition, biocatalyst made from 0.4% (*w*/*v*) alginate solution displayed better activity than those from 0.5% (*w*/*v*); this result could be also assigned to the difference in their size, since particles of lower alginate concentration were smaller. Therefore, the highest activity was exhibited using alcalase immobilized onto UAD 0.4% (*w*/*v*) alginate beads, with the maximal value of 2716.1 IU $g^{-1}$ support and the highest enzyme loading of 671.6 ± 4 mg $g^{-1}$ carrier in comparison to 2600.8 IU $g^{-1}$ and 592.3 ± 6.7 mg $g^{-1}$ for EE biocatalyst made from 2% (*w*/*v*) alginate solution.

### *2.5. Application of the Immobilized Alcalase in the Industrially Feasible Reaction System of Food Proteins Hydrolysis*

The performance of UAD and EE biocatalysts was investigated in the real food systems for hydrolysis of egg white and soy proteins. Figure 6 shows the degree of hydrolysis (DH) as a function of time for all biocatalysts. Obviously, the type of the alcalase beads and type of protein substrate affected the initial rate, extent, and pattern of hydrolysis. The degrees of hydrolysis of egg white protein achieved with alcalase-UAD 0.4% alginate and alcalase-EE alginate were 36.4 ± 0.83% and 35.9 ± 0.74% at 135 min and 165 min, respectively. On the other hand, free alcalase hydrolyzed 35.1 ± 1.125% of egg white protein in 75 min period which is in agreement with the previous work [33]. This means that with both biocatalysts it was possible to reach the same degree of hydrolysis, but UAD particles provided 1.3-fold higher rate of hydrolysis than EE biocatalyst; this can be ascribed to one or more general features of immobilized enzyme systems such as restricted accessibility of the substrate to the active sites of the enzyme caused by diffusional limitations, steric effects and enzyme structural changes following immobilization. The same conclusion was derived from hydrolysis of soy proteins with poorer performance of all alcalase biocatalysts in comparison to that in reaction with egg white proteins. Specifically, the overall degree of hydrolysis was around 26% accomplished after 120 min, 135 min, and 195 min for free alcalase, UAD and EE biocatalysts, respectively. For a sake of comparison, alcalase immobilized onto chitosan beads exhibited equilibrium degree of hydrolysis of 16.38 ± 0.989% with the same soy isolate substrate and under the same conditions as those used herein [33]. The immobilization of enzymes has been often accompanied by a decrease in enzyme activity, that seemed to be mainly due to diffusion limitations of the large substrate molecules inside the pores of the enzyme, which could be modulated by using carriers with higher pore diameter as reported in literature [17]. Thus, these results of proteolytic activity of the immobilized alcalase was contrary to the results reported for the alcalase immobilized on HMSS-$NH_2$-$Fe^{3+}$, which had even higher biocatalytic efficiency for bovine serum albumin proteolysis by 2.1 fold than the free enzyme. The higher activity of the immobilized enzyme was explained by the activation of alcalase by coordination with metal ions, which was advantageously changed the secondary structure of enzyme and was confirmed by FT-IR analysis [17]. According to the results of hydrolysis of both, egg white and soy substrates, the alcalase immobilized on UAD alginate beads seemed to present favorable catalytic performances in comparison to its EE counterpart.

From the industrial point of view, despite the lower reaction rate, the use of immobilized enzymes is economically viable since they can be recovered at the end of a hydrolysis cycle and reused, while the free enzyme must be inactivated to stop the reaction, generally by heating. Thus, the feasibility of the UAD 0.4% alginate in successive soy protein hydrolysis cycles was also evaluated during 8 cycles, spanning a period of more than 15 days, while storing the immobilized enzyme in 0.05 M Tris-HCl buffer solution (pH 8.0) containing 0.2 M $CaCl_2$ at 4 °C. The results are presented in Figure 6b. By fitting the retained activity trend with a linear function, it could be possible to evaluate the half-life of the biocatalyst (Figure 6c). It appeared that the activity gradually went down, showing a half-life of 3792.3 min (63.2 h) corresponding to around 16.8 batches. Namely, the immobilized alcalase retained activity during 7 subsequent hydrolysis cycles (1575 min), where after the loss of activity became more significant. This decrease in enzyme activity may be attributed to the leakage of enzyme during the hydrolysis, separating and washing processes

or to the enzyme deactivation by inhibition with substrate and products. Moreover, the enzyme deactivation by agitation could also contribute to the operational half-life. The immobilized alcalase was stable for at least 15 days of storage under mentioned conditions. The immobilized enzymes on alginate beads have been reported to be stable during the storage. For example, wet entrapped pectinase in alginate beads retained its initial enzymatic activity up to 11 weeks, whereas the lyophilized biocatalyst kept their original activity even after 8 months of storage [39]. Finally, it has been shown that the hydrolysis of soy protein with alcalase improved its nutritional content, techno-functionality and antioxidant activity [3,40]. However, the safety aspect of bioactive peptides should also be considered [41]. Although this preliminary results revealed that the alcalase immobilized on UAD alginate beads can be interesting for food proteins hydrolysis, further additional studies on hydrolysate and biocatalyst characterizations as well as the possible safety concerns of bioactive peptides must be performed to further improve the performance of the immobilized alcalase.




(**a**) (**b**) (**c**)

**Figure 6.** (**a**) Enzymatic progress curves of hydrolysis of egg white protein and soy protein isolate with alcalase immobilized on EE alginate beads and UAD 0.4% alginate. (**b**) The stability of the alcalase immobilized on UAD 0.4% alginate in repeated cycle using soy protein as substrate. Each cycle lasted for 225 min. Reaction condition: 1% (*w*/*w*) soy protein isolate aqueous solution (2.4 mg $mL^{-1}$, protein content), 50 °C, pH 8.0, stirring at 240 rpm. (**c**) Retained activity as a function of time.

## 3. Materials and Methods

### *3.1. Materials*

Alcalase® (protease from *Bacillus licheniformis*, Subtilisin EC 3.4.21.14) 2.4 L, sodium alginate of low viscosity, Tris-HCl buffer, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), azocasein, sodium hydroxide, trichloroacetic acid, trinitrobenzene sulfonic acid (TNBS), 2-mercaptoethanol, and hydrochloric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chicken egg white kindly provided from PKB "Inshri", Padinska Skela, was separated from the yolk and gently stirred without foam formation to provide homogeneous mixture. Soy protein isolate with a protein content of 90% was received from Sojaprotein, Serbia. All other chemicals used in this research were of analytical grade.

### *3.2. Preparation of Calcium Alginate Beads by Electrostatic Extrusion Technique*

Alginate beads were obtained by using electrostatic extrusion droplet generation [10]. Polymer solution of 2% (*w*/*v*) was prepared by dissolving low viscosity sodium alginate powder in distilled water. Then the suspension was agitated for 3 h by a magnetic stirrer (500 rpm) (Staufen, Germany). Spherical droplets were formed by extrusion of the polymer suspension through a blunt stainless-steel needle (22 gauge) using a syringe pump (Razel, Scientific Instruments, Stamford, CT, USA) and a 20 mL plastic syringe. Electrode geometry with the positively charged needle and a grounded hardening solution was applied. The potential difference was controlled at 6.5 kV by a high voltage DC unit (Model 30R, Bertan Associates, Inc., New York, NY, USA). The distance between the needle tip and the hardening solution (2% (*w*/*v*) $CaCl_2$ solution) was 2.5 cm while the flow rate of polymer solution was kept at 27.3 mL $h^{-1}$.

### *3.3. Preparation of Calcium Alginate Beads by Ultrasonic Spray Atomization System*

Sodium alginate solutions of 0.4 and 0.5% (*w*/*v*) concentrations were obtained by dissolving the low viscosity sodium alginate in a powdery form in distilled water. The solutions were mixed with a magnetic stirrer (350 rpm), until the complete dissolution of the alginate was reached after 1 h. The novel ultrasonic spray atomization system is custom made and configured as an ultrasonic spray atomization unit (for atomization of alginate solution) optionally coupled with a heater (for drying of alginate aerosol particles) and a glass column (for alginate aerosol gelation). An ultrasonic frequency change generator (Hoofddorp, The Netherlands) KCW-6TD, 220 V/50 Hz, Omron Profi Sonicwas used to apply a constant frequency of 1.7 MHz. The glass tube in which the heater was located at one end was connected to a compressor that allowed the flow of warm air, while the other end was divided into two parts. One part was connected directly to the device containing the sodium alginate solution over the plastic hose, while the other part through the plastic hose permitted the transport of dried particles, which were carried by the air stream to the vessel with the appropriate calcium chloride solution of 2% (*w*/*v*). The scheme of the apparatus for the preparation of calcium alginate submicrobeads by ultrasonic spray atomization method is shown (Figure 7).

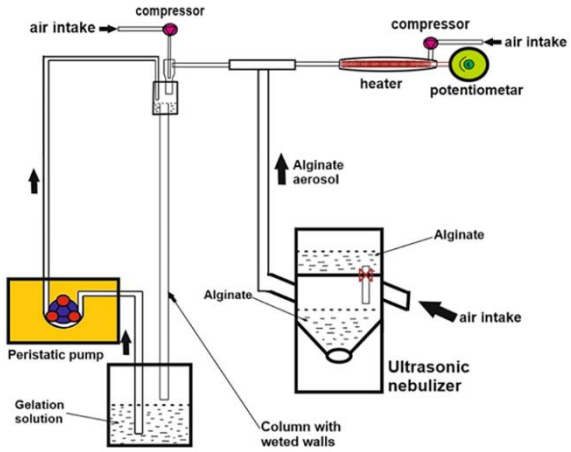



**Figure 7.** Scheme of the apparatus for the preparation of calcium alginate submicrobeads by utrasonic spray atomization method.

### *3.4. Immobilization Method*

The beads were treated with certain amount of EDAC (10–60 mg) in Tris-HCl buffer at pH in the range of 7.0 to 9.0 at 25 °C for 15 min to 24 h under gentle stirring. The effect of coupling buffer on the yield and immobilized alcalase activity was also studied by varying concentration of the Tris-HCl buffer between 10 and 200 mM. After a certain period, 0.33 IU to 5.32 IU of the alcalase was added in the solution and activation was performed for 1 to 48 h. The activated microbeads (0.5 g of wet weight) with the initial ratio enzyme-carrier 0.66–10.64 IU $g^{-1}$ were submerged into the enzyme solution at 25 °C under gentle stirring (150 rpm). The immobilization time was varied in the range 15 min to 24 h. At the end of this period, the produced derivative was washed with 50 mM Tris-HCl buffer, pH 8.5, followed by washing with distilled water after which it was stored at 4 °C in 50 mM Tris-HCl buffer, pH 8.5 before being used. Samples of the enzyme solution before and after the immobilization, together with the washing solutions, were taken for protein content and enzyme activity determination. The alcalase concentration was determined according to the Bradford method using bovine serum albumin (BSA) as a standard [42]. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduced into the coupling reaction mixture and the amount of enzyme in the

filtrate and in the washing solutions. The efficiency of immobilization was evaluated in terms of enzyme coupling yield. The enzyme coupling yield was calculated as follows:

$$\eta_{enz}(\%) = P_g / P_0 \times 100 \quad (1)$$

where $P_g$ is the immobilized amount of the alcalase and $P_0$ is the initial amount of the alcalase in the enzyme coupling solution determined by Bradford method.

### *3.5. Alcalase Activity Assay*

The alcalase activity was measured using azocasein as a substrate [43]. Assay mixture containing 75 µL of the enzyme solution or different mass of immobilized alcalase (0.01–0.2 g) and 125 µL of 2% (*w*/*v*) azocasein in 50 mM Tris–HCl (pH 9.0) was incubated for 30 min at 37 °C. The reaction was terminated by the addition of 600 µL of 10% (*w*/*v*) trichloroacetic acid. In order to remove the resulting precipitate, the assay tubes were cooled down in an ice bath prior to centrifugation for 10 min at 8000 rpm. Subsequently, 600 µL of supernatant was added to 700 µL 1 M NaOH and the absorbance at 440 nm was measured against a reference tube prepared separately for each sample by addition of trichloroacetic acid stop solution immediately after mixing the enzyme solution with the substrate. One unit of alcalase activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 1.0 in a 1-cm cuvette, under the above-mentioned assay conditions. The specific activities of the immobilized enzyme are expressed as unit per mg of protein immobilized on the support.

### *3.6. FT-IR Analysis*

Fourier transform infrared spectroscopy of dry samples was performed using Bomem MB 100 FT-IR spectrophotometer, applying the KBr disc method. Test samples consisted of 1 mg of sample mixed and grinded with 50 mg of potassium bromide and compressed into pallets at a pressure of 11 t for about a minute, using a Graseby Specac model: 15.011. The spectra were obtained in the wave number range between 4000 and 400 $cm^{-1}$ at 25 °C and at 4 $cm^{-1}$ spectral resolution.

### *3.7. Bead Size*

The size and size distribution of the alginate microbeads obtained by electrostatic extrusion were determined using particle size analyzer Mastersizer 2000 (Malvern Instruments, Worcestershire, UK), equipped with the Hydro 2000S dispersion unit. The mean diameter over volume was used as representative diameter. One of the common indicator of size distribution is the span defined as span of size distribution = (D90 − D10)/D50, representing how far the 10 percent and 90 percent points are apart, normalized with the midpoint. The mean size of alginate submicron-sized beads produced by ultrasonic spray atomization was measured by photon correlation spectroscopy, using Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., Malvern, UK) with the measurement range of 0.6 nm to 6 mm. The measurements of zeta potential were also performed by this instrument.

### *3.8. Scanning Electron Microscopy (SEM)*

The effect of alcalase on surface morphology of alginate microbeads was examined using a TESCAN Vega TS 5130MM scanning electron microscope (Brno, Czech Republic) at different magnifications. SEM analysis was performed at 20.0 and 10.0 kV.

### *3.9. Enzymatic Hydrolysis of Egg White Protein and Soy Protein Isolate with the Immobilized Alcalase*

The activity of the immobilized alcalase in the industrially feasible reactions was assayed by monitoring the hydrolysis of 1% (*w*/*w*) soy protein isolate aqueous solution (2.4 mg $mL^{-1}$, protein content) and 10% (*w*/*w*) aqueous solution of pretreated egg white (11.4 mg $mL^{-1}$, protein content determined according to the standard Kjeldahl method [44]. The hydrolysis was carried out in a 600 mL mechanically stirred batch reactor (Staufen,

Germany) with temperature and pH control. Prior to the enzymatic hydrolysis, the egg white protein solution was subjected to the thermal pretreatment at 75 °C for half an hour and afterwards, the solution was kept out at an ambient temperature to cool. After pH and temperature stabilization (about 20 min) at optimum conditions for alcalase (50 °C and pH 8.0), the hydrolysis reaction was initiated by adding the immobilized enzyme into egg white and soy protein solution, with stirring at 240 rpm [4]. The amounts of used immobilized enzymes were equivalent to the amount of free and corresponded to the activity of 9.98 IU and 0.53 IU for egg white and soy, respectively. The hydrolytic activity of the alcalase immobilized on UAD alginate beads towards soy protein isolate was tested during 8 cycles under the same conditions, and the operation time for each batch was 225 min. After each reaction cycle, the biocatalyst was removed by filtration, washing and storing between each experiment in 0.05 M Tris-HCl buffer solution (pH 8.0) containing 0.2 M $CaCl_2$ at 4 °C.

### *3.10. Determining of Degree of Hydrolysis*

The progress of the enzymatic hydrolysis was followed by monitoring the degree of hydrolysis (DH) by the TNBS method [45]. This method is basically a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines. The reaction takes place under slightly alkaline conditions and with the lowering of the pH is terminated.

The degree of hydrolysis (DH) is calculated according to the equation:

$$DH(\%) = h/h_{\text{tot}} \times 100 \quad (2)$$

where: $h$—the number of equivalent peptide bonds hydrolyzed at time $t$, $h_{\text{tot}}$—the total number of peptide bonds in the protein substrate in mmol $g^{-1}$ protein (meq $g^{-1}$ protein).

### *3.11. Statistical Analysis*

In this research, all experiments were carried out in triplicates and results were expressed as means with standard deviation. Analysis of variance, followed by the Tukey test was performed to compare the results. All the tests were considered statistically significantly at $p < 0.05$. All statistical analyses including calculations were conducted using MATLAB software (MATLAB R2014b., Natick, MA, USA). The graphs were created in OriginPro 8.5.

## 4. Conclusions

The work presented here has demonstrated novel simple and fast technique for alginate submicron beads production by employing the custom-made ultrasonic spray atomization system and compared it with the electrostatic extrusion in the hydrolytic reactions with standard and industrial food protein substrates. For these purposes, alcalase was effectively covalently immobilized onto calcium-alginate microbeads and submicron beads after surface modification by carbodiimide. The optimal conditions for the alcalase immobilization on the alginate microbeads activated with EDAC were 5.32 IU of alcalase, 50 mM Tris-HCl buffer pH 8.5, 30 min of activation by EDAC/beads mass ratio (10 mg/0.5 g) and 20 h immobilization time. The highest activity was exhibited with alcalase immobilized onto UAD 0.4% ($w/v$) alginate beads, with the maximal value of 2716.1 IU/g support and the highest enzyme loading of 671.6 ± 4 mg/g on the carrier in comparison to 2600.8 IU/g and 592.3 ± 6.7 mg/g for EE biocatalyst made from 2% ($w/v$) alginate solution. The immobilized enzyme onto UAD 0.4% ($w/v$) alginate beads provided better performance than micro-sized alginate counterparts in terms of egg white and soy protein hydrolysis. The biocatalyst can be reused in seven successive soy protein hydrolysis cycles with a little decrease in the activity. Therefore, there is potential to use this type of alginate submicron beads obtained by novel ultrasonic spray atomization technique for any enzyme immobilization.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2073-4344/11/3/305/s1, Figure S1: Effect of mass of the alcalase-alginate biocatalyst (immobilized alcalase) obtained by using one step and two-step conjugation protocol (with addition of 2-mercaptoethanol) on activity of the immobilized enzyme at two initial EDAC/microbeads mass ratio.

**Author Contributions:** Conceptualization, Z.K.-J.; M.J.; B.J.; M-Ž.; methodology, Z.K.-J.; B.J.; M.M.; V.Đ.; N.Š.; software M.J.; M.Ž.; Z.K.-J.; validation, M.J.; M.Ž.; B.J.; V.Đ.; Z.K.-J.; formal analysis M.J.; B.J.; Z.K.-J.; V.Đ.; investigation, M.J.; M.-Ž.; B.J.; M.M.; resources, B.B.; Z.K.-J.; M.M.; data curation, M.J.; M.Ž.; V.Đ.; N.Š.; writing—original draft preparation, M.J. and M.Ž.; writing—review and editing, Z.K.-J.; V.Đ.; supervision, Z.K.-J.; B.B.; project administration, Z.K.-J.; B.B.; funding acquisition, M.J.; B.B.; V.Đ.; Z.K.-J. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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