UDC 547.96:547.441.543.54
ISSN 1330-9862

scientific note

(FTB-1267)

Chemical Modifications on Proteins Using Glutaraldehyde

Carla J. S. M. Silva[1], *Fernanda Sousa*[1], *Georg Gübitz*[2] *and Artur Cavaco-Paulo*[1]*

[1]Department of Textile Engineering/Minho University, Guimarães, Portugal

[2]Department of Environmental Biotechnology, Graz University of Technology, Graz, Austria

Received: September 22, 2003
Accepted: February 16, 2004

Summary

In this work the effect of crosslinking the enzyme esperase (E.C. 3.4.21.62) and the proteins bovine serum albumin and casein with the bifunctional compound glutaraldehyde on molecular mass increase was studied. Two common techniques for measuring molecular mass of proteins were used: SEC and SDS-PAGE. These techniques revealed that the proteins bovine albumin and casein, when subjected to chemical crosslinking with glutaraldehyde, volume fraction 0.25 %, increased their molecular mass by 20- and 40-fold, respectively. It was also observed that M_r increased proportionally to the increase of glutaraldehyde concentration in the solution, and that the addition of glutaraldehyde should be done slowly, in small amounts, in order to attain bigger protein aggregates. When the proteolytic enzyme esperase was subjected to glutaraldehyde, no increase in its M_r was achieved. Several assumptions can be made to explain these results, the most reasonable being the low amount of free lysine groups available for crosslinking. This study confirms that glutaraldehyde is not an adequate crosslinker for esperase.

Key words: protease, proteins, crosslinking, glutaraldehyde, chromatography, SDS-PAGE

Introduction

The use of enzymes to treat textile fibres is being extensively studied in order to achieve more environmentally friendly processes. In the case of wool fibre, there are attempts to substitute the conventional chlorine treatment by an enzymatic process capable of providing the fabric with the same characteristics, like anti-shrinking and anti-pilling behaviour. In enzymatic wool treatment with proteases, the diffusion of the enzyme inside the wool fiber causes unacceptable losses of strength. It was thought that if the proteases were chemically modified in order to increase their molecular weight, their attack would be restricted only to the surface of the fibres, thus removing the cuticle, which is the main interest of this study.

Glutaraldehyde is a bifunctional compound mainly used in chemical modifications of proteins and polymers. This bifunctional compound links covalently to the amine groups of lysine or hydroxylysine in the protein molecules creating a structure more stable than that attained by the physical aggregation of protein molecules induced by the addition of salts, organic solvents, or non-ionic polymers. These solid aggregates are held together by non-covalent bonding and readily collapse and redissolve when dispersed in an aqueous medium (*1*). Thus, the chemical modification of proteins with crosslinking agents can be used for the reinforcement of the compact tertiary structures resulting in protein stabilisation against pH inactivation (*1*) and several approa-

* Corresponding author; Phone: ++351 253 510 280; Fax: ++351 253 510 293; E-mail: artur@det.uminho.pt

ches of chemical modification have also been used to increase the thermostability of proteases, like trypsin, α-chymotrypsin and subtilisin (2).

In the present work it is intended to study the behaviour of several proteins modified by glutaraldehyde, such as the formation of dimers and higher oligomers and the production of enzymatic aggregates with preserved activity. Proteins studied include bovine serum albumin, casein and the proteolytic enzyme esperase. Esperase is a modified subtilisin, used for finishing in textile industry. Alkaline proteases (or subtilisins) are one of the most important industrial enzymes, used primarily as detergent additives, accounting for nearly 60 % of total world enzyme sales (*3*).

Materials and Methods

Materials

The enzyme used in this study was the protease esperase, a modified subtilisin (E.C.3.4.21.62) (from Novozymes). The proteins bovine serum albumin and casein (from Sigma) were used as controls. All other reagents used were of analytical grade.

Procedure for the preparation of bovine serum albumin aggregates

A solution of 20 mg/mL of albumin was prepared (A_0) in 10 mM sodium acetate and 5 mM calcium acetate buffer, pH=7.5. Aggregation of the molecules was induced by slow addition of glutaraldehyde (from Aldrich, 50 % of fresh solution in water) to the clear solution under gentle stirring at 4 °C for 2 h. Several solutions were prepared containing 0.06, 0.13 and 0.25 % of glutaraldehyde in the solution, and the samples were labelled as $A_{0.06}$, $A_{0.13}$ and $A_{0.25}$, respectively.

Procedure for the preparation of casein aggregates

A solution of 5 mg/mL of casein was prepared (C_0) in 50 mM potassium phosphate buffer, pH=7.5. Aggregation of the molecules was induced by slow addition of glutaraldehyde (from Aldrich, 50 % fresh solution in water) to the clear solution under gentle stirring at 4 °C for 2 h. Several solutions were prepared containing 0.01, 0.02, 0.04, 0.08 and 0.25 % of glutaraldehyde in the solution, and the samples were labelled as $C_{0.01}$, $C_{0.02}$, $C_{0.04}$, $C_{0.08}$ and $C_{0.25}$, respectively.

Procedure for the preparation of enzyme aggregates

The enzyme aggregates were prepared using a solution of 20 mg/mL of the enzyme esperase and 50 μM antipain, a protease reversible inhibitor, in 10 mM sodium acetate and 5 mM calcium acetate buffer, pH=7.5 (E_0). The amount of 1 mM $CaCl_2$ was also added to the solution. A fresh solution of 1 % glutaraldehyde was added slowly under gentle stirring at 4 °C, until reaching the final volume fraction of 0.01, 0.02, 0.04, 0.06 and 0.20 %, the samples were labelled as $E_{0.01}$, $E_{0.02}$, $E_{0.04}$, $E_{0.06}$ and $E_{0.20}$, respectively.

Gel electrophoresis

To separate the proteins and to determine their molecular weights, SDS-PAGE was carried out using the Hoefer miniVe system from Amersham Pharmacia Biotech. The resolving gels (10 % acrylamide of about 1.5 mm thickness) were run at a constant voltage (120 V) and prepared according to the method originally described by Laemmli (*4*). The current was stopped when the bromophenol blue dye marker had reached about 1 cm from the bottom of the gel. Following electrophoresis, staining was carried out using Coomassie blue to observe the protein banding pattern on the gel. Phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used for calibration.

Size-exclusion chromatography

The protein size was determined by size-exclusion chromatography using a UV-detector at 280 nm and a Hi-Prep Sephacryl S-300 HR column (Amersham Pharmacia Biotech). The conditions of the assay were: room temperature; eluent: 50 mM phosphate, 100 mM KCl, buffer pH=6.5; flow: 2.5 mL/min and sample volume of 1 mL. Thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa) and carbonic anydrase (29 kDa) were used for calibration.

Degree of covalent modification

The method used for determining the modification of proteins at their amino groups is the modified assay of Morçöl *et al.* (*5*) in which the primary amines on proteins react with the sodium salt of trinitrobenzenesulfonic acid (TNBS). Unmodified (or native) proteins were used as standards in the experiments. The preparations of modified proteins and the protein standards were diluted in the concentration range of 0.05 to 1 mg/mL in 0.1 M borate buffer containing 0.15 M NaCl, pH=8.0. Two mL of the samples were mixed with 50 μL of 30 mM aqueous TNBS solution and the mixture was incubated for 30 min at room temperature. The blank for the assay consisted of 2 mL of buffer. The absorbances of modified and unmodified proteins were read against the blank at 420 nm and the data were plotted as a function of increasing protein concentration. The degree of covalent (irreversible) modification at amino groups was calculated using the formula:

$$\text{covalent modification fraction}/\% = [(A-B)/A] \times 100 \quad /1/$$

where A and B are the slopes of the unmodified standard and modified protein, respectively, as determined from the absorbance data at 420 nm in the linear regime.

Enzyme assay

The activity of proteases was measured at 37 °C following the increase in absorbance at 660 nm with 0.65 % of casein solution in 50 mM phosphate buffer (pH=7.5) as substrate. After incubation of 1 mL of diluted enzyme for 10 min at 37 °C with 5 mL of casein solution, the reaction was stopped by the addition of 5 mL of 110 mM TCA solution in water, and the precipitate was removed by filtration and centrifugation. The amino acids pro-

duced were analysed at 660 nm, taking DL-tyrosine as standard. One unit of activity is defined as the amount of the enzyme that hydrolyses casein to produce the colour equivalent to 1 μmol of tyrosine per minute, at pH= 7.5 and 37 °C (colour by the Folin&Ciocalteu's reagent).

Total protein concentration was determined by the Bradford (*6*) method, using bovine serum albumin as standard.

Results and Discussion

To accomplish this study, two proteins were chosen as model proteins because they are widespread and cheap. These proteins were a globular protein, bovine serum albumin (BSA), having a relative molecular mass close to 66 kDa and a flexible protein, casein (CAS), whose relative molecular mass is near 23 kDa. This milk protein was also chosen because it has a relative molecular mass close to that of the esperase, the enzyme that was intended to be studied.

Preparation of casein aggregates

The formation of casein aggregates was verified by the analysis of the chromatogram attained by SEC. The results are shown in Fig. 1.

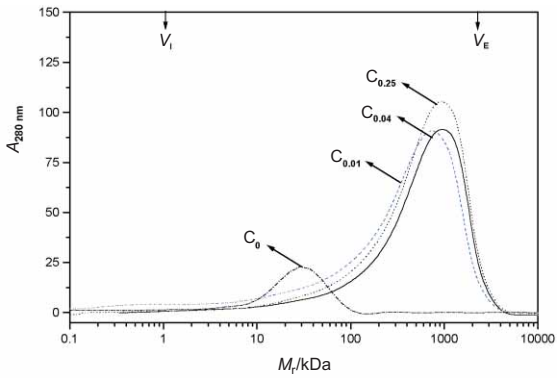

Fig. 1. Size-exclusion chromatography elution patterns of the native (C_0) and modified casein on 50 mM potassium phosphate buffer, pH=7.5. Modified samples were labelled as $C_{0.01}$ – casein solution with 0.01 % GTA; $C_{0.04}$ – casein solution with 0.04 % GTA and $C_{0.25}$ – casein solution with 0.25 % of GTA). The scale was modified from elution time (t_E) to molecular mass (in Da) using the calibration equation:

$$\log M_r = 7.85815 - 1.65159 \cdot ((t_E \cdot 2.5)/103.62)$$

As it can be seen in Fig. 1, after adding just 0.01 % GTA to the solution, an aggregate with high molecular mass is formed (curve $C_{0.01}$ on the chromatogram). This aggregate is about 40-fold the native casein size (C_0). Adding higher concentrations of GTA does not change the chromatogram profile significantly once this aggregate appears in the same chromatogram zone, very close to the column exclusion volume. It was expected that with the addition of low GTA concentrations, the formation of dimers or other oligomers of lower M_r occurred. Instead, big agglomerates were formed. Because this was not expected to occur, complementary assays were performed using SDS-PAGE. In this technique, an anionic detergent is used (sodium dodecylsulphate – SDS) to disrupt secondary and tertiary structures of protein molecules and weak interactions among them.

As can be seen in Fig. 2, defined multimers of casein are formed with the increase of glutaraldehyde concentration. At the lowest fraction of glutaraldehyde (0.01 %) (lane B in Fig. 2) it is possible to observe the formation of higher complexes that are trapped at the interface of the stacking gel and the running gel. The dominant band is shifted to a position corresponding to the trimeric form of casein. However, higher complexes appear as well, migrating more slowly in the gel. The dominant band starts to be smoother and it is possible to see the higher multimeric complexes trapped at the interface of the gels. In lane E (0.08 % GTA), all the casein complexes got trapped in the stacking gel. This pattern of crosslinking was not detected in size-exclusion chromatography, where all samples were presented as a multimeric complex of about 40 times the weight of casein. This may be due to the action of SDS that disrupts aggregated proteins which were not linked by covalent bonds, whereas in HPLC those formations are eluted in a non-disrupted form.

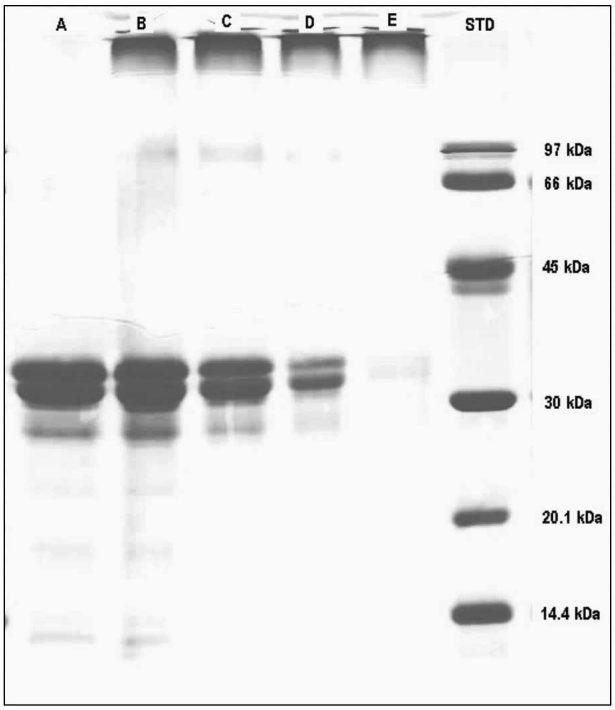

Fig. 2. SDS-PAGE of the samples of casein. Lanes: **A** – no addition of glutaraldehyde (native casein), **B** – casein with 0.01 % GTA, **C** – casein with 0.02 % GTA, **D** – casein with 0.04 % GTA, **E** – casein with 0.08 % GTA and **STD** – molecular mass markers

Preparation of albumin aggregates

In Fig. 3 the chromatogram attained by SEC to BSA is presented. Analysing this figure it may be seen that with the addition of GTA volume fraction 0.06 % to the protein solution, dimer formation occurs, *i.e.* two unmodified albumin molecules aggregate, having thus twice the molecular mass of native (unmodified) BSA. This molecular mass increases gradually with the increase of

final GTA concentration in the solution, seen in Fig. 3 by the curve shifts towards higher molecular mass from $A_{0.06}$ to $A_{0.25}$. When φ(GTA)=0.25 % is added to the solution (curve $A_{0.25}$ on the chromatogram), a big protein agglomerate is formed, having about 20-fold the size of unmodified albumin, and elutes near the exclusion column volume, determined by blue dextran. It is interesting to see that when this same concentration of GTA was added all at once (curve $A_{0.25*}$ on the chromatogram), the chromatographic profile was very different, presenting two main inflexions of the curve. It is believed that the first peak corresponds to the GTA that has not reacted with the protein. It is well known that this compound can promote self-oligomerization, explaining the molecular mass near 1 kDa.

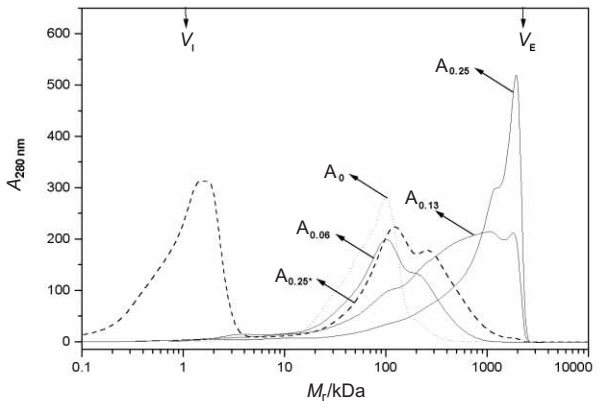

Fig. 3. Size-exclusion chromatography elution patterns of the native (A_0) and modified albumin on sodium and calcium acetate buffer, pH=7.5. Modified samples were labelled as $A_{0.06}$ – albumin solution with 0.06 % GTA; $A_{0.13}$ – albumin solution with 0.13 % of GTA and $A_{0.25}$ – albumin solution with 0.25 % GTA. The scale was modified from elution time (t_E) to molecular mass (in Da) using the calibration equation:

$$\log M_r = 7.85815 - 1.65159 \cdot ((t_E \cdot 2.5)/103.62)$$

In the second inflexion of the curve it is possible to see the peak of free BSA and its dimers (that is, BSA increased twice). This data leads us to the assumption that GTA reacts promptly with free lysine groups of proteins and, when in excess, it partially polymerises to give oligomers. For this reason, to attain protein oligomers of high molecular weight, it is advisable to add the bifunctional reagent slowly, in small amounts. The mechanism of the formation of multimers may be a two-step process, first the reaction of a monomer or oligomer with the crosslinker, and second, the reaction of this oligomer/monomer containing a crosslinker with a monomer lacking a crosslinker (a »free« monomer). This reaction may be a covalent linking type or just an electrostatic one. When GTA is added all at once, it links to all available lysine groups and then, perhaps by some phenomenon of steric hindrance, it polymerises to give GTA oligomers.

Preparation of enzyme aggregates

Similar experiments were conducted with esperase, a commercial subtilisin from Novozymes. In the chromatographic study performed with esperase, the GTA concentrations were kept below 0.20 %, in order to keep the enzyme active (data not shown).

In Fig. 4 the chromatographic profile for esperase modification by GTA is presented. Analysing this figure, it is seen that no increase in molecular weight occurs, despite the use of antipain, a protease inhibitor, to prevent autoproteolysis.

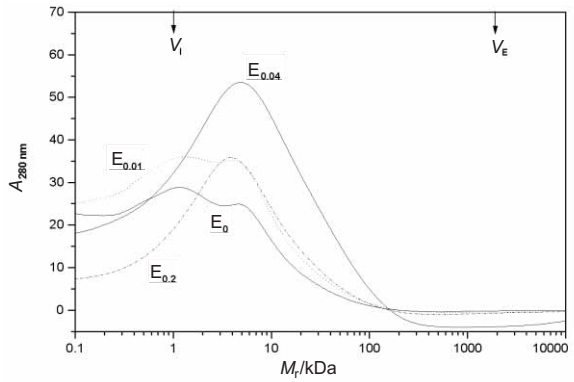

Fig. 4. Size-exclusion chromatography elution patterns of the native (E_0) and modified esperase on sodium and calcium acetate buffer, pH=7.5. Modified samples were labelled as $E_{0.01}$ – esperase solution with 0.01 % GTA; $E_{0.04}$ – esperase solution with 0.04 % GTA and $E_{0.20}$ – esperase solution with 0.20 % GTA. The scale was modified from elution time (t_E) to molecular mass (in Da) using the calibration equation:

$$\log M_r = 7.55643 - 1.43295 \cdot ((t_E \cdot 2.0) / 98.062)$$

The low concentration of GTA added in order to maintain the enzyme active may have had an influence on the poor enzymatic aggregation verified. Another plausible explanation is that esperase, being a commercial preparation, contains other compounds used as stabilisers that may have reacted preferentially with GTA. The amount of total protein present in the enzymatic preparation is in the order of 7 %, a value considered normal when compared to other commercial preparations, but indicative of the high amount of stabilisers and/or additives in the solution. It is, therefore, important that all amines or other compounds that may react with GTA should be removed prior to aggregation, in order to enhance the efficiency of GTA crosslinking.

Extent of crosslinking

In Fig. 5 the degrees of covalent modification of amino groups, calculated by the modified TNBS method, are presented for both proteins and esperase. The correlation coefficients of the plots were 0.98 or higher for both native and modified proteins.

In the case of casein, with low amounts of GTA (0.01 %), the degree of covalent modification was (74±4) %. Adding more GTA had only a slight effect on this degree, confirming the results of SEC and SDS-PAGE. For BSA, this increase in the crosslinking degree was less pronounced, and with 0.25 % of GTA added, it reached the value of (69±3) %. When the concentration of GTA was doubled, this value reached (80±1) %, and was still increasing. Once again, this test confirmed the re-

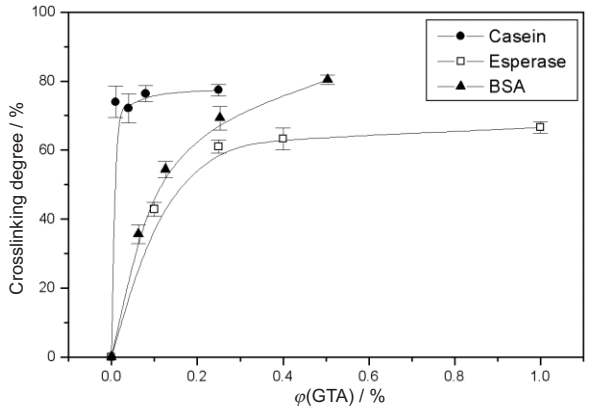

Fig. 5. Extent of crosslinking of BSA, casein and esperase as a function of glutaraldehyde (GTA) concentration

sults attained by SEC, showing that the formation of aggregates with BSA is a slower process than with casein.

Values in the same magnitude were also obtained by Bigi *et al*. (*7*), who found that with 0.25 % of GTA, the degree of crosslinking on gelatine films was about 85 % and increased to near 100 % when GTA concentrations above 1 % were used.

For esperase, it may be seen that the degree of covalent modification of amino groups using the bifunctional reagent glutaraldehyde occurs in a much slower way. With 0.25 % of GTA added to the enzymatic solution, the degree of covalent modification achieved for esperase was (61±2) %, this value being 16 % less than that attained for casein. The maximum crosslinking degree achieved for esperase was (66±2) %, when 1.0 GTA was used. This crosslinking degree was attained in the enzymatic preparation, which contains about 7 % of protein (*i.e*. enzyme), so it may correspond to the modification of other amino groups present in solution or other substances that may have reacted with GTA.

The number of available amino groups able to interact with GTA in each tested protein was investigated (Table 1). The proteins albumin and casein have 59 and 25 free amino groups, respectively, which are able to link covalently to glutaraldehyde (these free amino groups are originated from the ε-amino groups of lysine residues and a terminal α-amino group).

Table 1. Content of lysine residues in the proteins tested (from Protein Data Bank (*8*))

Protein	Lysine residues
esperase (P29600)	5
casein (P02663)	25
bovine serum albumin (CAA76847)	59

With regard to the protease esperase, it has only 5 available lysine residues (see Table 1). This fact, by itself, explains the poor aggregation verified with this enzyme. The problem of the non-accessibility of amino groups to be attacked by glutaraldehyde should not be a valid explanation since the ionised side-chains, like those of Lys and Asp, tend to be on the exterior of the enzymes and able to interact with the solvent (*9*).

Conclusions

The results of this paper confirm that the number of free lysine groups is a key issue in the formation of soluble aggregates when glutaraldehyde is used as the bifunctional reagent for crosslinking. In proteins with a high amount of free lysine residues, glutaraldehyde crosslinking constitutes an effective way for the formation of multimers. The different reactivity of glutaraldehyde on BSA and casein can be associated with the specific conformation of each protein. Casein is a relatively small protein with a flexible open structure, and thus the access of GTA to its lysine residues is facilitated compared to BSA, a globular protein, which has restricted accessibility of lysine residues to react.

It was found that the increase in M_r was gradual with the increase of final glutaraldehyde concentration in the solution. Interestingly, the way in which GTA was added was also important. It was confirmed that to attain protein oligomers of high molecular mass it is advisable to add the bifunctional reagent slowly and in small amounts. The explanation for this may reside in the fact that GTA reacts promptly with the available lysine residues, and then, perhaps by some steric hindrance, it self-polymerises to give GTA oligomers.

SDS-PAGE confirmed the aggregate formation attained in chromatography. It also showed that despite the formation of dimers and trimers, it is not always possible to see their existence by chromatography because they elute in a non-disrupted form, which means at the same elution time. Note that this chromatographic study was conducted without the addition of SDS to the elution buffer.

The modified TNBS method also confirmed the results of SEC and SDS-PAGE, showing the quick agglomerate formation for casein, even with low amounts of GTA.

For esperase, TNBS method showed a much slower reaction between GTA and the enzyme. Actually, maximum covalent modification of amino groups attained for esperase was 66 %, when a high concentration of GTA was used (1 %). It is thought that this value may correspond to the covalent modification of amino groups of other compounds possibly present in the enzymatic solution. It was seen that GTA concentrations of above 0.20 % increased high losses of enzymatic activity. For this reason, the chromatographic study performed with the enzyme used concentrations below that value. This study revealed that no agglomerate formation was found for esperase using this bifunctional compound. A valid explanation for this fact should be the low amount of lysine groups available for crosslinking in esperase.

These chromatographic results show that glutaraldehyde is not an adequate crosslinker for this enzymatic class. Other bifunctional compounds, able to interact with other enzyme reactive groups, should be used to increase its molecular mass to the desired values.

Acknowledgements

The authors are grateful for the funding by Fundação para a Ciência e Tecnologia (Portugal), *via* PhD fellowship, ref. PRAXIS XXI SFRH/BD/1292/2000.

References

1. L. Cao, F. Rantwijk, R. Sheldon, *Org. Lett. 2–10* (2000) 1361–1364.
2. Z. He, Z. Zhang, M. He, *Process Biochem. 35* (2000) 1235–1240.
3. C. G. Kumar, H. Takagi, *Biotechnol. Adv. 17* (1999) 561–594.
4. U. K. Laemmli, *Nature, 227* (1970) 680–685.
5. T. Morçöl, A. Subramanian, W. Velander, *J. Immunol. Methods, 203* (1997) 45–53.
6. M. Bradford, *Anal. Biochem. 72* (1976) 248–254.
7. A. Bigi, G. Cojazzi, S. Panzavolta, K. Rubini, N. Roveri, *Biomaterials, 22* (2001) 763–768.
8. Protein Data Bank: *http://www.rcsb.org/pdb.*
9. N. Price, L. Stevens: *Fundamentals of Enzymology: The Cell and Molecular Biology of Catalytic Proteins*, Oxford University Press, NY (1999) pp 47–110.

Unakrsno vezivanje proteina uporabom glutaraldehida

Sažetak

U radu je ispitivan utjecaj unakrsnog vezivanja enzima esperaze i proteina albumina iz goveđeg seruma i kazeina s glutaraldehidom da bi se dobila povećana relativna molekularna masa. Za mjerenje relativne molekularne mase proteina upotrijebljeni su poznati postupci SEC i SDS-PAGE. Korištenjem tih postupaka nađeno je da proteini goveđeg seruma, albumin i kazein, unakrsno vezani s 0,25 %-tnom otopinom glutaraldehida, povećavaju svoju molekularnu masu za 20 odnosno 40 puta. Ujedno je opaženo da se molekularna masa proporcionalno povećava s povećanjem glutaraldehida u otopini te da se dodavanje treba provoditi polagano i u malim količinama kako bi se postigli veći proteinski agregati. Kada se proteolitičkom enzimu esperazi dodao glutaraldehid, nije se povećala molekularna masa. Postoji nekoliko pretpostavki da bi se objasnio taj rezultat, a najvjerojatnije je da enzim sadrži vrlo malo lizinskih ostataka koji se mogu koristiti za unakrsno vezivanje. Ovim je radom potvrđeno da glutaraldehid nije adekvatno sredstvo za unakrsno vezivanje esperaze.