A C T A A L I M E N T A R I A P O L O N I C A

Vol. XII (XXXVI), No. 2 1986

DANUTA CZAJKOWSKA
ANNA WIĄCEK-ŻYCHLIŃSKA
BARBARA JĘDRYCHOWSKA
REGINA SAWICKA-ŻUKOWSKA

SOME SUBSTANCES PRESENT IN FUNGAL PROTEIN PREPARATIONS AS INTERFERING FACTORS IN THE DETERMINATION OF PROTEIN BY THE LOWRY METHOD

Institute of the Fermentation Industry, Warsaw

Key words: protein assay, colorimetric method, fungal protein preparation.

Study was made of the interference with the protein determination using the Lowry procedure, caused by starch, pectin, carboxymethylcellulose and of their enzymic degradation products as well as of other substances contained in the media used for the production of fungal protein preparations. The interfering effect of these substances was investigated prior to and after their pretreatment with 1 M NaOH at 100°C for 10 min. It was found that the investigated substances affect the protein results in a different measure, and that their pretreatment enhances and sometimes reverses the interfering action.

INTRODUCTION

Since some few years, at the Institute of the Fermentation Industry in Warsaw studies are made of the production of fungal protein preparations by culture on solid media (ca. 60% humidity). The main components of media are: coarse grain meal, milling waste, potatoes, dried sugar-beet pulp, apple pomace, mineral salts ($(NH_4)_2HPO_4$, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, NH_4NO_3, $Ca(NO_3)_2$) and urea. In the course of growth, fungal enzymes cause degradation of some complex components of medium. For effective observation of protein biosynthesis, a fast analytical method is required. We selected the method of Lowry et al. [16], which enables big series of analyses and is relatively simple and accurate (exact to 5 μg of protein). Moreover, the method enables the determination of the contents of water- and NaOH-soluble proteins as well as of proteins precipitated by trichloroacetic acid, acetone or other organic solvents [9, 16]. According to Raimbault and Alazard [18], in the assay of protein in fungal protein preparations (also by the method of Lowry et al.)

the analysed samples have to be pretreated with 1 M NaOH at 100°C for 10 min.

Literature data indicate that, apart from its simplicity and accuracy, the Lowry assay has certain drawbacks. Foremost among these are the nonlinearity of the standard curve and interference by various substances with the results. The causes for the nonlinearity of the standard curve are not yet fully elucidated [21]. The effect of various substances on the results has already been observed by the authors of the method [16], who specified the maximal concentration of some compounds still leaving the results of analysis unaffected. Also studies have been made of the effects of buffers [7, 14, 19], detergents and especially Triton X-100 [14, 15], EDTA, glycine, mercaptoethanol, sodium citrate, Tris-HCl, acetylacetone, penicillin and streptomycin [2], glycerol [4], thiol compounds [12], sugars [5, 10, 11], hexosamines [3], nucleotides, nucleosides, pyrimidine and purine bases [20], fatty acids and fats [8]. A detailed list of compounds affecting the results of the Lowry assay is given in Peterson's monograph [17].

The effects of all chemical compounds listed above have been studied under standard assay conditions without any pretreatment. Only Bonitati et al. [5] have applied in their study of interference by carbohydrates a pretreatment with 0.5 M and 1 M NaOH, and with 0.6 M and 1.8 M HCl; using a sugar concentration of 2 mg/cm^3, they demonstrated a frequent pretreatment-induced considerable interference with the protein assay results.

In order to eliminate this interference with the Lowry assay, a number of modifications have been introduced [1, 10, 12]. Some of them are reported in Peterson's monograph [17] and in a review article by Worowski and Ostrowska [22].

The available literature contains no information about the effect of starch, pectin, carboxymethylcellulose and other compounds present in fungal protein preparations, on protein levels assayed by the Lowry method. Likewise, the effect of pretreatment of these substances with a hot NaOH solution is not fully known. Accordingly, we investigated the effect of various substances present in fungal protein preparations, before and after pretreatment of samples with a hot NaOH solution (according to the procedure for sample preparation, proposed by Raimbault and Alazard, [18], on protein levels measured by the Lowry assay.

MATERIAL AND METHODS

1. Reagents and other substances: 1 M NaOH; copper reagent obtained by mixing 50 cm^3 of reagent A (2 g Na_2CO_3 in 100 cm^3 of 0.1 M NaOH) with 1 cm^3 of reagent B (0.5 g $CuSO_4 \times 5\ H_2O$ in 100 cm^3 of 1% sodium

citrate); Folin-Ciocalteu reagent according to Lowry et al. [16]; bovine serum albumin (Fluka AG); mineral salts, urea, soluble starch, maltose, saccharose (all purum p.a., from POCh, Gliwice), galactose purum p.a. (Xenon, Łódź);anhydrous glucose purum p.a. (Koch-Light Lab. Ltd.); dextrins (ZPZ, Łomża); apple pectin and its pectinolytic degradation products (ZPOW "Pektowin", Jasło).

2. "Spekol" spectrophotometer (Kar Zeiss, Jena).

3. Experimental procedure. A bovine serum albumin solution (100 μg/cm³ H_2O) was used for the estimation of the interference by the investigated chemicals with protein determination by the Lowry procedure. To the albumin solution, addition was made of an appropriate aqueous solution of each investigated substance, whereupon the Lowry assay was applied for albumin determination (protein level before the pretreatment of the substance). An analogous solution of the investigated substance in NaOH (1 g/100 cm³ 1 M NaOH), heated at 100°C for 10 min *), was added to the albumin solution and the Lowry assay was carried out (protein level after the pretreatment of the substance).

Lowry assay: To 1 cm³ of the albumin solution (100 μg of protein), containing an appropriate amount of the investigated substance, 5 cm³ of the copper reagent were added. After thorough mixing, the mixture was left at room temperature for about 40 min, and then its absorbance was read at 650 nm, against the reagent blank.

Protein content in the sample was read from a standard curve (10 to 400 μg/cm³).

RESULTS AND DISCUSSION

The standard curve for protein level determination by the Lowry method is shown in Fig. 1. Whereas the direct plot is nonlinear, in the logarithmic system (as also demonstrared by Stauffer, [21]) it is linear and is described by the equation: $\log A = -2{,}566 + 0.923 \log C_b$
where: A — absorbance, C_b — protein concentration in μg/cm³. The statistical characteristic the absorbance results for 60 samples (100 μg of bovine serum albumin (1 cm³) are as follows: arithmetic mean $\bar{x} = 0.201$; variance $s^2 = 8.05 \times 10^{-6}$; standard deviation $s = 0.0028$; coefficient of variation $v = 1{,}4\%$.

The interference by starch and dextrins, before and after pretreatment with a hot NaOH solution, is shown in Fig. 2. It is evident that starch (before and after pretreatment) and dextrins (before pretreatment) did not affect the protein determination. On the other hands, pretreated

*) This pretreatment refers to the procedure for sample preparation, proposed by Raimbault and Alazard [18].

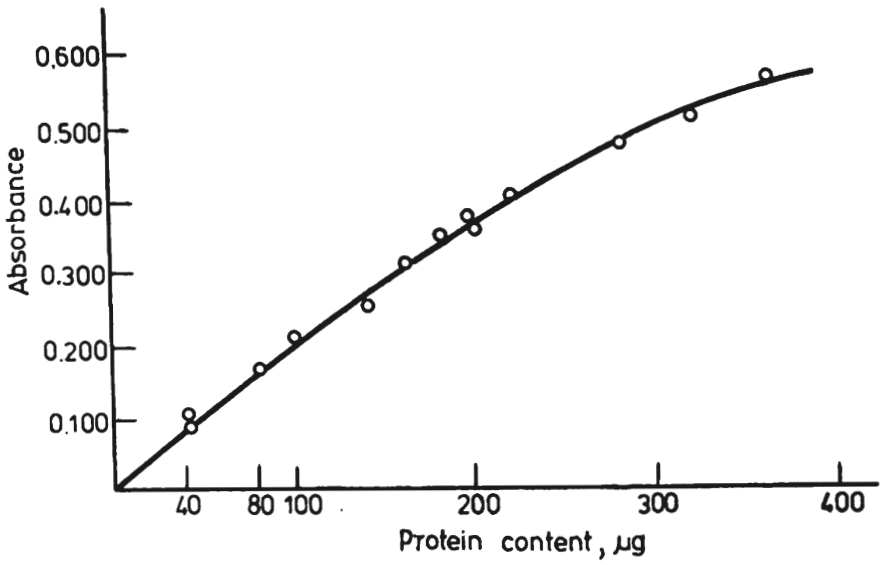

Fig. 1. Standard curve for protein (bovine serum albumin) determination by the Lowry method

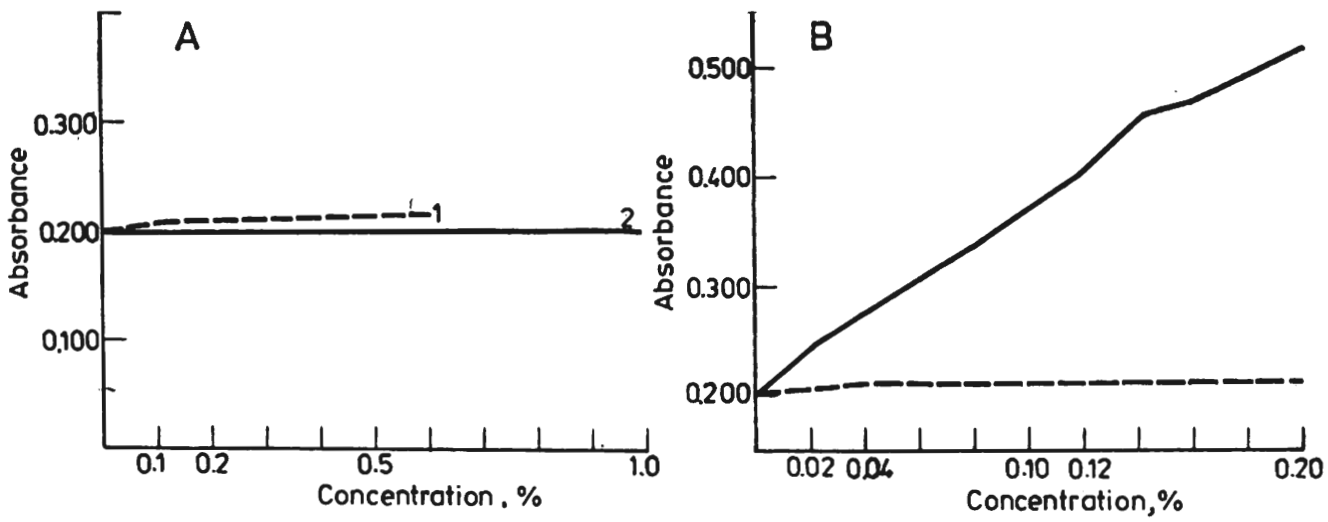

Fig. 2. Effect of starch and dextrins (A — untreated, B — pretreated) on the results of protein determination by the Lowry method; 1 — starch, 2 — dextrins

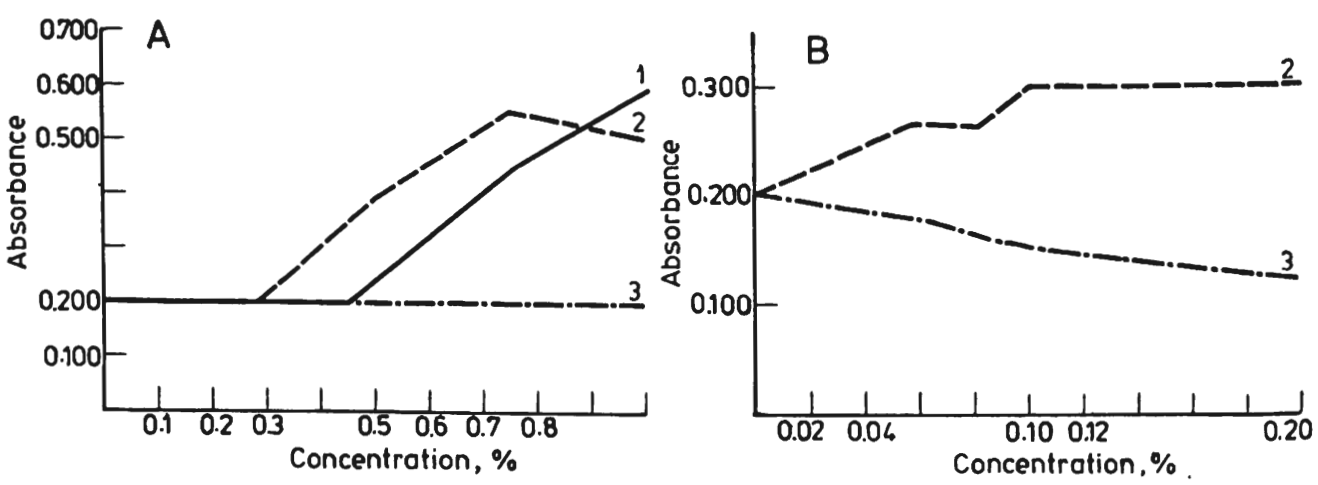

Fig. 3. Effect of pectin and urea (A — untreated, B — pretreated) on the results of protein determination by the Lowry method; 1 — pectin, 2 — products of enzymic degradation of pectin, 3 — urea

dextrins greatly increased the protein results; the interference became manifest at a 0.02% concentration, to attain 250% at a 2% concentration.

The interference by pectin and urea is illustrated in Fig. 3. Pectin and its pectinolytic degradation products (at concentrations up to 0.45% and 0.3%, respectively), when not pretreated, did not interfere with the protein results; at higher concentrations, the protein results were overestimated, the effect of the pectin hydrolysate — as compared with pectin — being more pronounced. After the pretreatment of pectin, the formation of a jellylike substance prevented the absorbance measurement. The pretreatment of the pectin hydrolysate increased the interfering effect (at a 0.2% concentration, the protein result rose by about 50%). Not pretreated urea failed to affect the protein results, even at concentrations of 1-5%. The pretreatment of urea caused a decrease in the absorbance readings, beginning from an about 0.02% concentration; at a 0.2% urea concentration, the protein result was by about 45% lower.

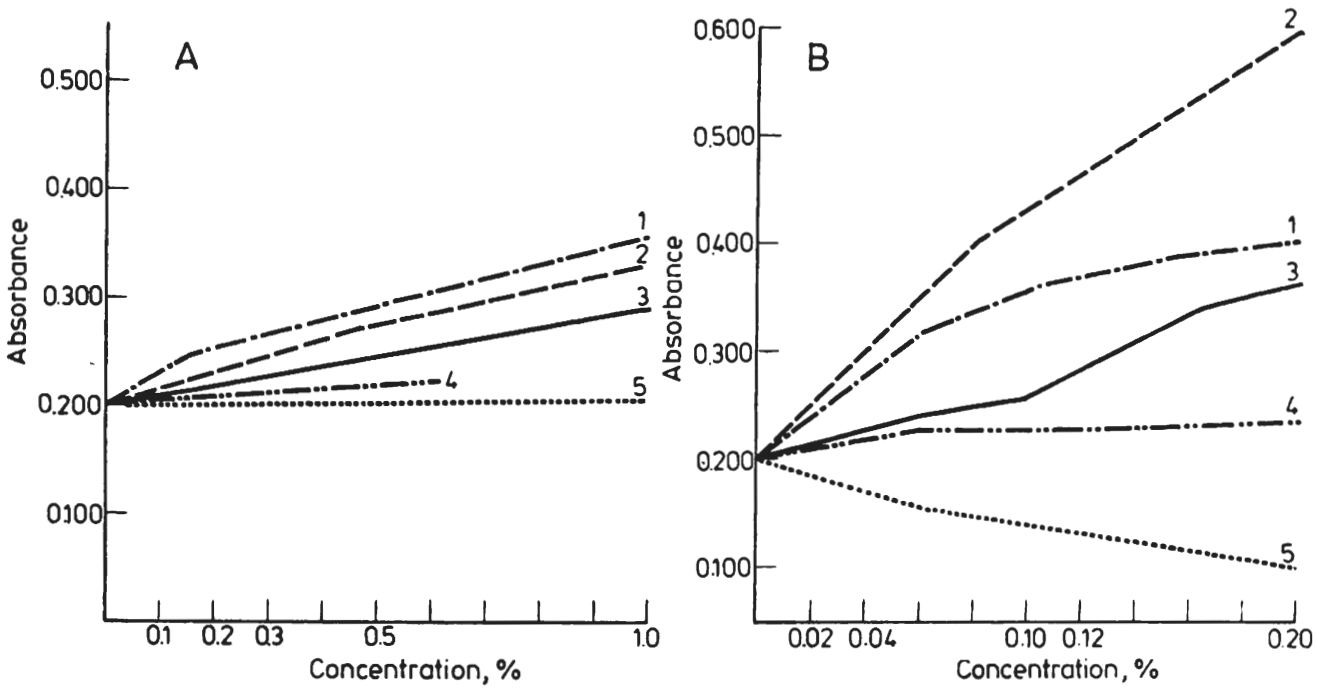

Fig. 4. Effect of saccharides and carboxymethylcellulose (A — untreated, B — pretreated) on the results of protein determination by the Lowry method; 1 — galactose, 2 — saccharose, 3 — glucose, 4 — carboxymethylcellulose, 5 — maltose

The results concerning saccharides and carboxymethylcellulose are given in Fig. 4. Before the pretreatment, glucose, galactose and maltose increased the absorbance readings, beginning from a 0.1% concentration; the effects of carboxymethylcellulose and saccharose were practically nil. The pretreatment of glucose, galactose and maltose increased the absorbance readings, starting from a 0.02% concentration; at a 0.2% concentration, the protein result increase by 90, 120 and 300%, respectively. The effect of pretreated carboxymethylcellulose remained practically nil. Pretreated saccharose decreased the absorbance readings; at a 0.2% concentration the protein result was understimated by 50%. The single effect

of saccharose, differing from that of other sugars and leading to lower absorbance readings, is explained by Gerhardt and Beevers [11].

Fig. 5. shows the results dealing with the effect of mineral salts. The presence of sodium nitrate at concentrations up to 5% did not affect the absorbance readings. As concerns $Ca(NO_3)_2$ and K_2HPO_4, precipitation of protein (at concentrations of 0.5 and 1%, respectively) caused an opalescence making the assay imposible. The remaining mineral salts studied decreased to a different degree the protein results at concentrations exceeding 1%.

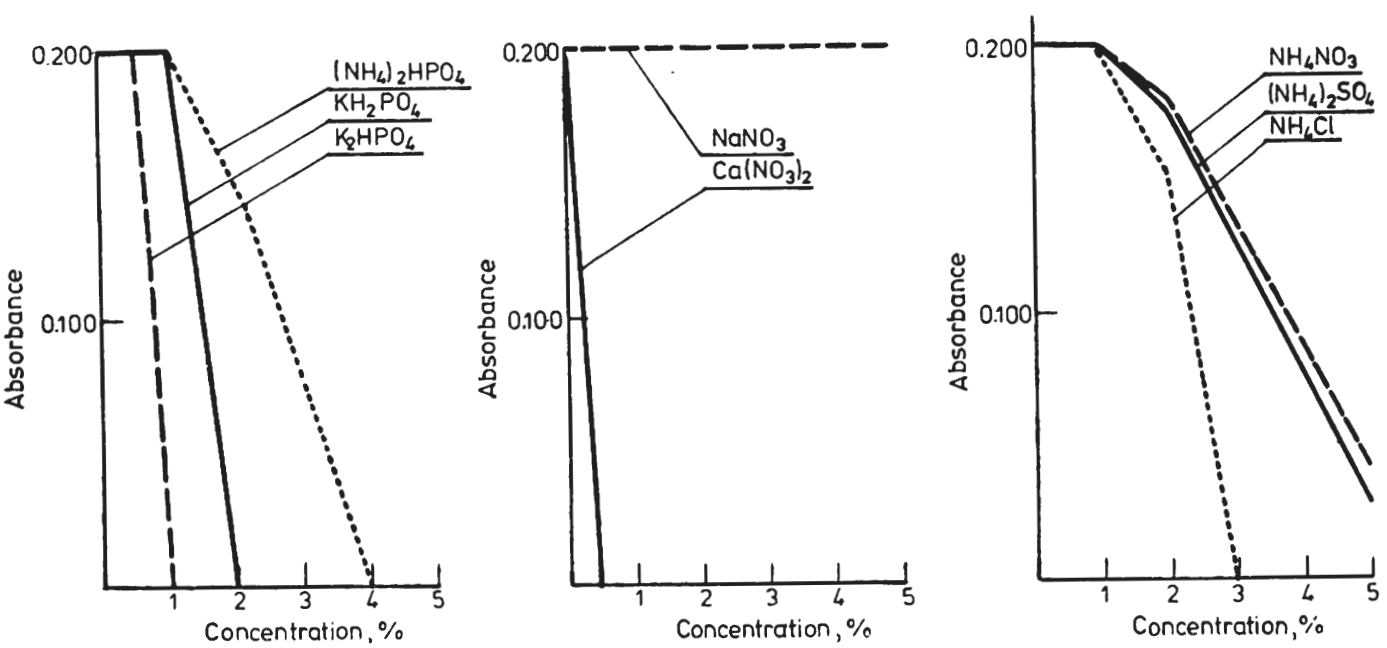

Fig. 5. Effect of mineral salts on the results of protein determination by the Lowry method

Literature data as well as our own protein assays by methods other than the Lowry procedure indicate that the amounts of protein in fungal media and its contents in the resulting fungal protein preparations are between 10-25%. In this connectio, prior to the protein determination by the Lowry method, the samples of these materials must be diluted 1200-2000 times; consequently, all components of these materials, which could interfere with the assay results, are diluted to the same extent, and thus their harmful effect greatly decreases. Our preliminary analyses have shown that in case of most investigated media and preparations the Lowry method proves to be appropriate, except for fungal protein preparations obtained on media with high contents of straw, apple pomace and sugar-beet pulp; namely, in these cases the protein results are overestimated and suitable corrections have to be made.

CONCLUSIONS

1. In the Lowry procedure, starch, dextrins, carboxymethylcellulose, saccharose and urea (all at concentrations below 1%) do not interfere with the protein determination.

2. Most of investigated salts decrease to a different degree the protein results, at concentrations exceeding 1%.

3. Glucose, galactose and maltose (at concentrations over 0.1%) as well as pectin and its enzymic degradation products (at concentrations exceeding 0.45 and 0.3%, respectively) increase the protein results.

4. The pretreatment of the above-mentioned substances with 1 M NaOH at 100°C for 10 min enhances and sometimes reverses their interference with the protein determination.

5. The chemical composition of the applied fungal culture media and of the resulting fungal protein preparations as well as the determinations of their protein contents by methods other than the Lowry procedure indicate that the Lowry assay is appropriate for the protein estimation in most cases, including materials preheated in 1 M NaOH at 100°C for 10 min; the media containing large amounts of straw, apple pomace and sugar-beet pulp as well as the resulting protein preparations are an exception to this rule.

LITERATURE

1. Bejer E. M.: Lab. Delo 1976 (10), 590.
2. Bensadoun A., Weinstein D.: Anal. Biochem., 1976, **70**, 241.
3. Berg D. H.: Anal. Biochem., 1971, **42**, 505.
4. Blümel P., Uecker W.: Anal. Biochem., 1976, **76**, 524.
5. Bonitati J., Elliot W. E., Miles P. G.: Anal. Biochem., 1969, **31**, 399.
6. Chou S. C., Goldstein A.: Biochem. J., 1960, **75**, 109.
7. Cookson C.: Anal. Biochem., 1978, **88**, 340.
8. Eichberg J., Mokrash L. C.: Anal. Biochem., 1969, **30**, 386.
9. Final recommendation on the measurement of cellulase activities Commision on Biotechnology IUPAC 1983.
10. Gaál D., Holezinger L.: Acta Biochim. Biophys. Acad. Sc. Hung., 1975, **10**, (3), 201.
11. Gerhardt B., Breevers H.: Anal. Biochem., 1968, **24**, 337.
12. Higuchi M., Yoshida F.: Anal. Biochem., 1977, **77**, 542.
13. Higuchi M., Yoshida F.: Agric. Biol. Chem., 1978, **42** (9), 1669.
14. Ji T. H.: Anal. Biochem., 1973, **52**, 517.
15. Letoy K., Jesudian Ch. R.: Anal. Biochem., 1975, **69**, 632.
16. Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem., 1951, **193**, 265.
17. Peterson G. L.: Anal. Biochem., 1979, **100**, 201.
18. Raimbault M., Alazard D.: Eur J. Appl. Microbiol. Biotechnol., 1980, **9** (3), 199.
19. Rej R., Richards A. H.: Anal. Biochem., 1974, **62**, 240.
20. Shepard G. R., Hopkins P. A.: Anal. Biochem., 1963, **35**, 1548.
21. Stauffer C. L.: Anal. Biochem., 1975, **69**, 646.
22. Worowski K., Ostrowska H.: Wiad. Chem., 1982, **36**, 29.

Manuscript received: April, 1984
Authors address: 02-532 Warszawa, Rakowiecka 36

D. Czajkowska, A. Wiącek-Żychlińska, B. Jędrychowska, R. Sawicka-Żukowska

WPŁYW WYBRANYCH SUBSTANCJI WYSTĘPUJĄCYCH W GRZYBOWYCH PREPARATACH BIAŁKOWYCH NA WYNIKI OZNACZEŃ ZAWARTOŚCI BIAŁKA METODĄ LOWRY'EGO

Streszczenie

Prześledzono wpływ skrobi, pektyny, celulozy i produktów ich rozkładu enzymatycznego oraz innych związków chemicznych używanych jako składniki podłóż i występujących w grzybowych preparatach białkowych, na wyniki oznaczeń zawartości białka metodą Lowry'ego, zaproponowaną przez Raimbaulta i Alazarda (18) Wpływ wyżej wymienionych substancji określano bez i po wstępnym traktowaniu roztworem 1 M NaOH w temp. 100°C przez 10 min.

Stwierdzono, że skrobia i dekstryny (rys. 2), karboksymetyloceluloza i sacharoza (rys. 4) oraz mocznik (rys. 3), nie traktowane wstępnie gorącym roztworem NaOH, w stężeniach do 1% nie wpływają na wyniki oznaczeń zawartości białka metodą Lowry'ego. Większość spośród przebadanych soli mineralnych, w stężeniach powyżej 1%, zaniża w różnym stopniu odczyty ilości białka badaną metodą (rys. 5). Glukoza, galaktoza i maltoza w stężeniach powyżej 0,1% (rys. 4) oraz pektyna i produkty jej enzymatycznego rozkładu w stężeniach wyższych niż odpowiednio 0,45 i 0,3%, nie traktowane wstępnie gorącym roztworem 1 M NaOH, (rys. 3) zawyżają odczyty absorbancji. Wstępne traktowanie roztworem NaOH w temp. 100°C przez 10 min wyżej wymienionych substancji organicznych na ogół potęguje i często zmienia ich wpływ na odczyty zawartości białka w próbce. Na przykład glukoza, galaktoza i maltoza, poddane wstępnemu ogrzewaniu w roztworze NaOH, zawyżają odczyty absorbancji począwszy już od stężenia 0,02% (rys. 4), natomiast mocznik począwszy od tego samego stężenia (0,02%) wyraźnie zaniża wyniki oznaczeń zawartości białka (rys. 3).

W związku z dużym rozcieńczaniem próbek przed wykonaniem oznaczenia (od 1000 do 2000 razy) a tym samym znacznym zmniejszaniem się stężenia substancji towarzyszących, mogących wpływać na wyniki analizy, metoda Lowry'ego zaproponowana przez Raimbault a i Alazard a (18) jest przydatna i pozwala uzyskiwać porównywalne wyniki zawartości białka dla większości grzybowych preparatów białkowych. W niektórych jednak przypadkach, na przykład przy stosowaniu podłóż zawierających znaczne ilości słomy i/lub wytłoków jabłkowych oraz wysłoków buraczanych obserwuje się wyraźne zawyżenie wyników oznaczeń białka, co wskazuje na konieczność zmodyfikowania metody (np. uwzględnianie odpowiednich poprawek).