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Influences of Different Dissolving Method of Succinic Anhydride on the Succinylation Level of some Proteins

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The influences of different dissolving methods of succinic anhydride (SA) were examined in the succinylation for some proteins. Sample proteins were BSA, casein, egg white albumin, and myofibrillar proteins and sarcoplasmic proteins obtained from fish. The pH value of the protein solution was maintained at 8.5 during the gradual addition of SA (Method A), or the pH value was adjusted at 8.5 after the dissolution of all required SA (Method B). The relationship between log(SA/Protein,w/w) and the succinylation level in percentage revealed a smooth S-shaped curve by method A, whereas that by method B was not smooth but had an inflection point. Method B succinylation consumed greater amounts of SA than method A to obtain an equal succinylation level. Both the appearance of the inflection point in the succinylation curve and a large consumption of SA in method B were caused by the isoelectric point precipitation of the proteins in the succinylation process.

1 Introduction

In the previous paper[1], we reported the method to control the succinylation level of some proteins. In this method, the pH value of protein solution was kept in a constant range during every addition of partitioned SA, because succinylation proceeds under mild alkaline pH range. This succinylation method, however, is very difficult to keep the constant pH range of the solution. If no significant differences exist between the succinylation method in which the calculated SA was dissolved all at once to protein solution and the usual succinylation method employed in the previous paper, it would be more simple and easy without such a tedious procedure. This succinylation method, however, is assumed to be significantly affected on the succinylation level, because pH value of the protein solution will decrease by addition of a large amounts of SA, and accordingly the protein will be denatured. We conducted some experiments, therefore, to examine the influences of dissolving method of SA on the succinylation level of some proteins.

2 Materials and Methods

2.1 Sample Proteins

The proteins used in the examination were three commercially available proteins, BSA (Wako Pure Chemicals), casein (Merck), EWA (Wako Pure Chemicals), and two kinds of fish proteins, Mf-P

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The following abbreviations were used for the chemicals and sample proteins in this paper: SA, succinic anhydride; BSA, bovine serum albumin; EWA, egg white albumin; Mf-P, myofibrillar proteins; Sp-P, sarcoplasmic proteins.

and Sp-P prepared from ordinary dorsal muscle of rudder-fish *Girella punctata*. Both Mf-P and Sp-P were prepared according to the methods described in the previous paper[1].

2.2 Methods of Succinylation

Succinylation of the protein was carried out by dissolving SA to the sample protein solution (4mg protein/ml) according to the following two different methods: Groninger's method[2], in which pH value was maintained at 8.5 during every addition of a partitioned SA (Method A), and all of calculated SA was dissolved at once to the protein solution and subsequently pH value was adjusted at 8.5 (Method B). Both 7.5N and 1N NaOH were used for pH adjustment. Thus the succinylated protein solutions were allowed to stand overnight at 5℃, and subjected to the measurement of succinylation level.

2.3 Calculation of the Succinylation Level

Succinylation level was calculated by Kakade and Liener's method[3] unless otherwise noted, and Habeeb's method[4] was also employed in some experiment. The protein content was measured by the micro-biuret method[5]. Extinction coefficients of the sample proteins were identical to those of the previous paper[1].

3 Results and Discussion

3.1 Succinylation of BSA

Sample BSA was separately succinylated by methods A and B, and the relations of succinylation level and log (SA/BSA,w/w), namely the succinylation curve, was defined in the previous paper as shown in Fig. 1.

The shapes of two succinylation curves differed from each other; the curve by method A was a smooth S-shaped curve, whereas that by method B was inflected curve. The latter curve was situated on the right side of the former, and these curves indicated that the succinylation by method B needs greater amounts of SA than that in method A for obtaining an equal succinylation level.

3.2 Possible Reasons for the Appearance of an Inflection Point in the Succinylation Curve for BSA

The main reason why the inflection point was appeared in the succinylation curve of BSA by method B was speculated to be as follows; pH value of the BSA solution decreases with dissolution of SA, and then three-dimensional structure of BSA would be changed by a mutual interaction between positively charged amino groups. To inspect this hypothesis, pH value of the BSA solution was measured during stepwise addition of SA. Thus obtained pH variation curve is shown in Fig. 2, together with the succinylation curve of BSA shown in Fig. 1.

As shown in Fig. 2, pH value of the BSA solution decreased gradually with SA addition, but no large pH fluctuation was seen around the inflection point of the succinylation curve. The pH value around

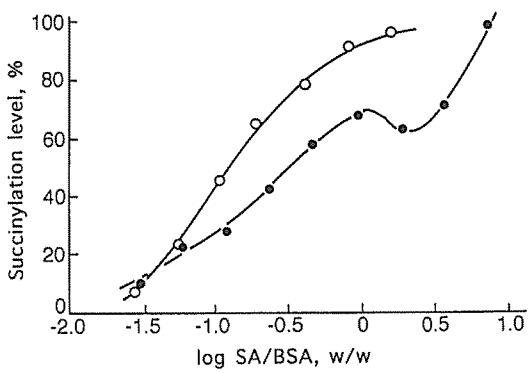

Fig. 1. Influences of different dissolving methods of SA on the succinylation level of BSA.
The pH value of the BSA solution was maintained at 8.5 during every addition of a slight amount of SA (method A, — ○ —), and dissolving calculated SA all at once to the BSA solution and subsequently pH value was adjusted at 8.5 (method B, —●—).

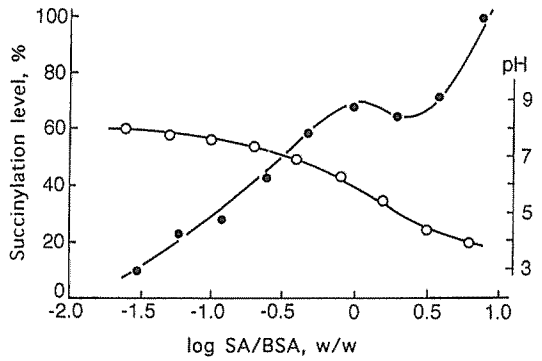

Fig. 2. The relations of succinylation curve and pH variation curve of BSA. Succinylation was carried out by the method B.
—●—, succinylation level; —○—, pH

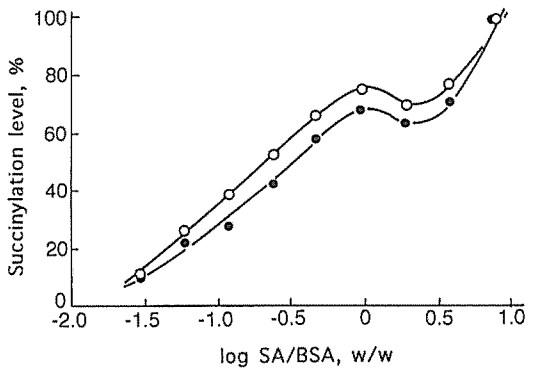

Fig. 3. Comparison of the succinylation curves between with and without dialysis. Succinylation was carried out by the method B using BSA.
—○—, non-dialyzed; —●—, dialyzed.

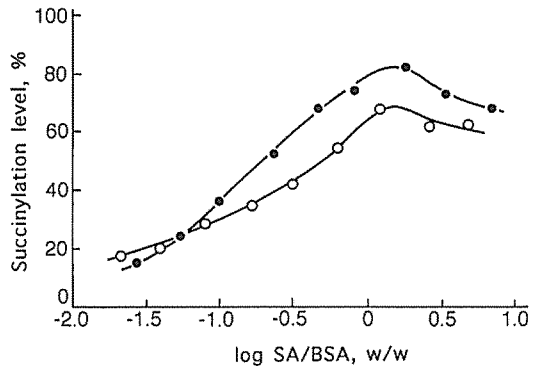

Fig. 4. Influences of different measuring methods of succinylation level of BSA.
—○—, calculated by Lys content[3]; —●—, calculated by amino group content[4].

the inflection point was about 5 as seen in Fig. 2, and this pH was adequately approximated to the isoelectric point of BSA, 4.9. As a result, the reason for the appearance of an inflection point in the succinylation curve was, therefore, thought depending on the isoelectric point precipitation. In addition, the increase in succinylation level at pH regions lower than the isoelectric point was thought to be as follows: first of all, pH value of the BSA solution decreased lower than the isoelectric point with increasing of added SA, and the amount of positively charged amino groups increased on the whole surface of the BSA molecules. The total surface area of BSA molecule would be unfolded by mutual repulsion among positively charged amino groups, accordingly. As a result, it was thought reaction between BSA and SA was increased.

Alternatively, contaminated amino group-containing substances in protein, and determination method of succinylation level involved with the appearance of an inflection point in the succinylation curve by method B. The influence of contaminated substances on the succinylation curve wasexamined by dialyzing of succinylated BSA solution against phosphate buffer (pH 8.5), and the results were shown in Fig. 3. In addition the influences of measuring method on succinylation level were investigated by employing Habeeb's method[4] instead of Kakade and Liener's method[3], and their results were compared in Fig. 4.

As show in these results, a little difference in succinylation curves were observed in both Figs. 3 and 4. It seems coexisting contaminants influenced a few, and also measuring methods of succinylation level did a few. It will be concluded that the possible reason for the appearance of an inflection point was depending on the isoelectric pointprecipitation of BSA.

3.3 Succinylation of Casein and EWA

EWA and casein were succinylated separately by

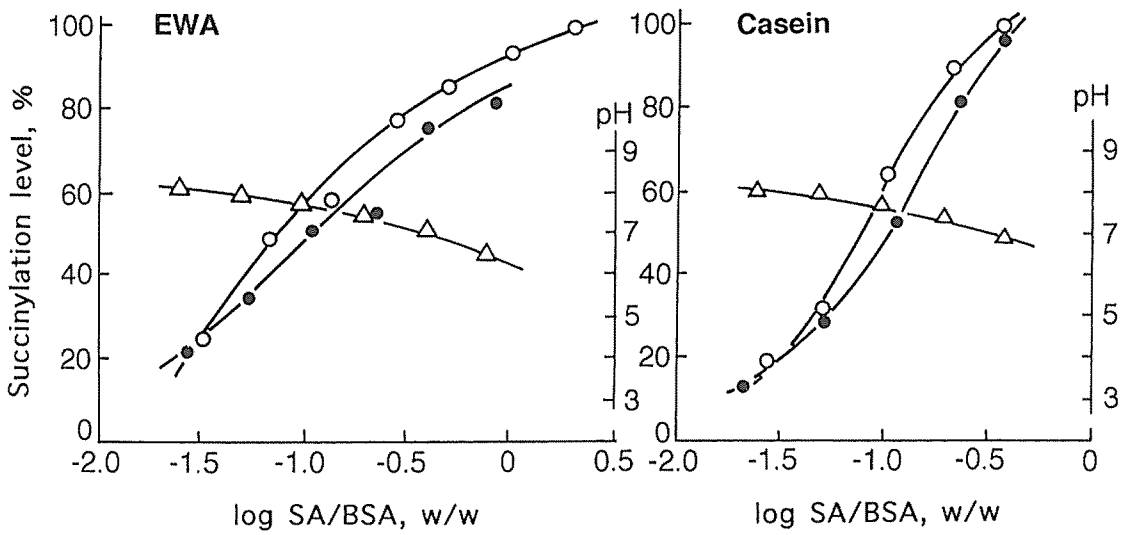

Fig. 5. Influences of different dissolving methods of SA on the succinylation level of EWA and casein.
—○—, succinylation by method A; —●—, succinylation by method B; —△—, pH variation.

both methods A and B, and their succinylation curves and pH variations are summarized in Fig. 5.

As apparent from Fig. 5, the shapes of two different succinylation curves resembled each other in either EWA or casein, but slightly large amount of SA was needed for method B than method A to obtain the equal succinylation level. These results from EWA and casein were similar as from BSA.

The inflection point appeared in BSA, however, was not seen in either EWA and casein. The reason why the inflection point did not appear was thought to be as follows: Isoelectric point of both proteins is 4.6, and log (SA/protein) which correspond to their isoelectric points from their succinylation curve were situated over 100% of succinylation level, and accordingly, the inflection point did not appeared in either EWA or casein.

3.4 Succinylation of Mf-P and Sp-P

Two kinds of fish proteins, Mf-P and Sp-P, were succinylated by methods A and B, and their results were compared in Fig. 6.

As seen in these results, the properties of Mf-P

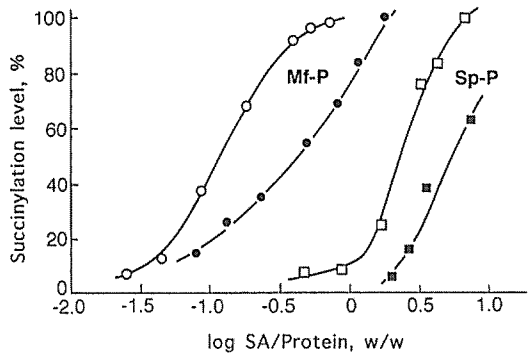

Fig. 6. Influences of different dissolving methods of succinic anhydride on the succinylation level of two kinds of fish proteins, Mf-P and Sp-P.
—○—, —□—, succinylation by method A; —●—, —■—, succinylation by method B.

and Sp-P for succinylation were quite different from each other. That is, a greater amount of SA was needed for Sp-P than Mf-P in any succinylation methods of A and B. For example, the amount of SA which was required for 50% succinylation level of Sp-P was 18 times more than that of Mf-P in method A, and 13 times more in method B.

To clarify the reasons for Sp-P of the lower reac-

tion for succinylation than Mf-P, the Sp-P was succinylated after dialysis once against running water. Some contaminants including amino group were speculated to coexist in the Sp-P solution, and the prepared Sp-P solution was dialyzed to eliminate some speculated contaminant. If there exists some contaminants including amino group, they might react with added SA and the effective SA used for the succinylation of Sp-P would be consumed. Consequently, succinylation of Sp-P is thought to be lower than Mf-P. The result of this experiment is shown in Fig. 7.

Two succinylation curves of Sp-P with or without dialysis nearly coincided with each other, as shown in Fig. 7. Accordingly, the possibility of coexistence of low molecular substances including amino group was too low. However, it was considered that high molecular substances which was not removed by dialysis still remained in the Sp-P solution. Hence, the reasons why Sp-P was harder for the reaction of succinylation than Mf-P still remain to be clarified.

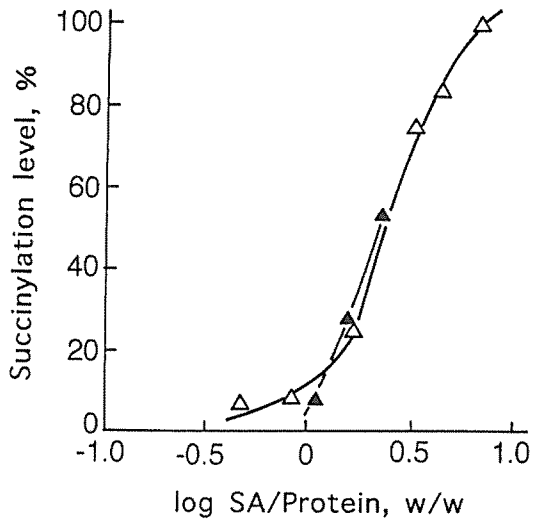

Fig. 7. Comparison of the succinylation curves by method A between with and without dialysis of Sp-P.
—△—, non-dialyzed; —▲—, dialyzed.

On the other hand, the property of protein for succinylation was found to be noticeably different in both methods A and B, as seen in Figs. 1, 5 and 6. That is, method B succinylation consumed greater amounts of SA than method A to obtain a same succinylation level. The same results were obtained for BSA, EWA, Casein, and two kinds fish proteins, Sp-P and Mf-P.

From the results in this study, it was concluded the pH value of protein solution cause a physicochemical change in the dissolved protein. Therefore, SA addition and pH adjustment must be balanced to avoid the fluctuation of the solution pH value.

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タンパク質のサクシニル化に及ぼす無水コハク酸の溶解方法の影響

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タンパク質のサクシニル化に当たって無水コハク酸(ＳＡ)を加えた後にｐＨ調整した時(Ｂ法)の結果を，ｐＨを微調整しながらＳＡを加えた時(Ａ法)の結果と比較した。供試タンパク質は牛血清アルブミン(ＢＳＡ)，卵白アルブミン，カゼイン，魚肉(メジナ)の筋形質タンパク質および筋原線維タンパク質の５種類である。ＢＳＡの場合，ＳＡとタンパク質の重量比の対数値(log ＳＡ/protein)とサクシニル化率の関係(サクシニル化曲線)は，Ｂ法では大きな変曲点が見られたのに対してＡ法では見られなかった。他のタンパク質では変曲点は見られなかった。サクシニル化曲線に変曲点が現れた理由は，ＳＡの添加によってｐＨが下がり等電点沈殿を起こしたためであることが分かった。