The Research of Small Molecule Soybean Polypeptide Precipitated by TCA

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Abstract. Trichloroacetic acid (tca) makes macromolecular peptides expose more hydrophobic groups and have precipitation effect. It's widely used in the desalination and concentration of protein and peptide. This experiment use the alkaline protease enzymolysis the soybean protein isolated and makes the products as raw material, then do the high performance liquid chromatography (hplc) analysis of tca precipitation before and after enzymolysis liquid. Macromolecular peptidesore more than 10000 completely precipitated. Results show that the precipitation rate of more than 3000 u reached 62.01%, the molecular weight of 3000-1000 u is about 37.71%.When the molecular weight is less than 1000 u, the polypeptide precipitation rate drops to 22.51%. For the peak belonging 420 u, the main reason is that tca have strong absorption peak and a small amount of impurity absorption peak.

Introduction

Trichloroacetic acid (tca), because it can change the protein conformation into exposing more hydrophobic groups, can be regarded as a kind of protein and polypeptide precipitant used in all aspects of the related studies [1]. As an accepted protein precipitator in practical application, the dosage of tca is obtained by the gradient method. The weight range of the precipitation in the specific molecular, sedimentation rate didn't have actual research. The public usually think tca precipitation of tens to hundreds of thousands of peptides macromolecular components are completely [2] - [3], but the case of small molecular peptides have no detailed document to prove. For example, we use the tca/acetone solution to do the protein sample concentration and desalination. Now it has been clearly proposed that tca destroys the structure of the macromolecular and it was not easy to be clear, so it is mainly used for the enrichment and desalination of small polypeptides. For small molecule polypeptides, the molecular weight range and settling rate is not accurate. In-depth understanding of tca molecular weight range of ATP eptide precipitation and precipitation rate are good for correctly tca use [4], as far as possible reduces the experimental error. This experiment is based on hydrolysates of soy protein isolated, the hplc analysis of the before and after tca precipitation sample liquid. Then we

compare its molecular weight distribution, and conclude the tca precipitation scope of small molecular peptide molecular weight and its precipitation rate.

Materials and Methods

Materials

Soybean protein isolated (spi), Alcalasa enzyme, tca, casein, copper sulfate, potassium sodium tartrate, formaldehyde, phenolphthalein, potassium acid phthalate, NaOH and HCl, acetonitrile, trifluoroacetic acid and methanol.

Sample: cytochrome C (Mw = 12500 Da), aprotinin (Mw = 6511.44 Da), Bacitracin (Mw = 1422.69 Da), Gly Gly - Tyr - Arg (Mw = 451.48 Da)

High performance liquid chromatography (LC-20 a Japanese), ultraviolet spectrophotometer (UV - 2450, Japan), fully automatic kjeldahl nitrogen determination apparatus (KjelFlex K - 360).

Methods

The Preparation of Samples. [5, 6] SPI (2.5g) contains about 90 % high-quality protein. Distilled water was added to form 5% solution and placed in a boiling water bath for 10 min. Then it was cooled to 53 ℃. NaOH solution was used to adjust the pH (=8) value of the initial reaction. Alkaline protease (3642 u/g) was joined to react for 30min, 1h, 2h and4h. The reaction solution was adjusted ph=4.5 by HCl. After that, the solution was immersed in a boiling water bath for 15 min to inactivate the enzyme. The reaction solution was then centrifuged at 4000 r/min for 20 min, and the supernatant was filtered to obtain the spi hydrolyzate.

The Determination of Total Protein Content. [7] GB 50095-2010, the content of protein was 88.57%.

Degree of Hydrolysis. [8] Formaldehyde titration.

The Yield of Peptides. [9, 10] The preparation of standard curve (casein), Biuret method is to determine the rate of peptide.

Fractionation of SPI Hydrolysates by HPLC. The chromatographic conditions [11]:7.8 ×300 mm (Waters) with a C18 cartridge as guard column. Mobile phase: acetonitrile/water/trifluoroacetic acid and 0.1/45/55 (V/V), the flow rate was 0.5 mL/min. Detection was carried out at 220 nm. Standard sample: cytochrome C (Mw = 12500 Da), aprotinin (Mw = 6511.44 Da), Bacitracin (Mw = 1422.69 Da), Gly Gly - Tyr - Arg (Mw = 451.48 Da)

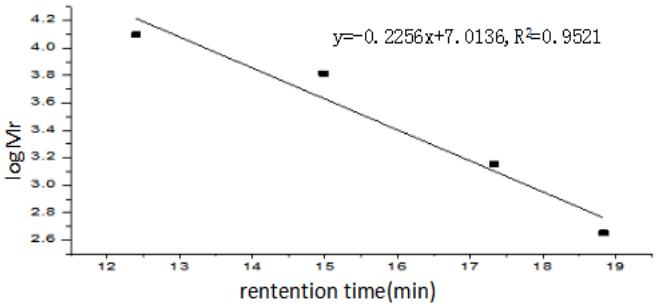

Figure.1 HPLC standard sample figure

Samples analysis: The sample solution 5 ml combined with 5 ml20 % of TCA shocked 10 s and stood for 10 min, centrifuged 20 min under 8000 r/min, separated out clear liquid. In addition, we let the same enzymolysis liquid mix with pure water (1:1), ditto. Finally, the same amount of samples was taken by a syringe, respectively passed

the filtration membrane and analyzed. Under the condition of the chromatographic analysis, the chromatographic data of the sample was taken into the standard curve equation to calculate to get the peptide molecular weight and its distribution range [12].

Results and Discussion

Different Time of Digestion

The Impact on the Degree of Hydrolysis. From Fig. 2, at the beginning of the enzyme solution, the degree of hydrolysis increased sharply with the extension of time.1h later, hydrolysis degree tended to be stable. After 4 h, DH value reached 16.37%.

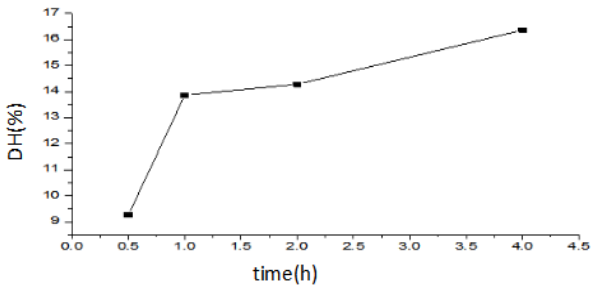

Figure 2. Variation of degree of hydrolysis with time

The Impact on the Peptide Yield. From Fig. 3, as time extended, because of a small amount of peptide reconnection, the degree of peptides drops. The last peptide yield is 77.30%.

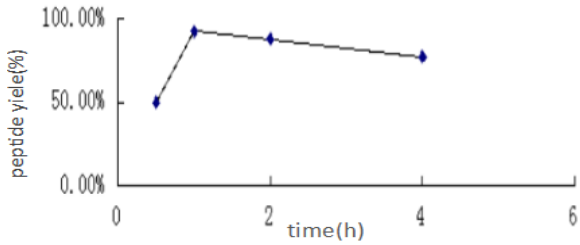

Figure 3. Enzymatic hydrolysis time and peptide yield

The Analysis of HPLC

From Fig. 4 , the molecular weight distribution of the sample without tca is 13800 u - 426 u .After the precipitation ,a range become 2950 u - 426u .There were two peaks at 23.5and 32.15 min. From the Fig. 5, we concluded that 10% TCA solution has two big peaks at 23.5 and 32.1 at 220 nm.

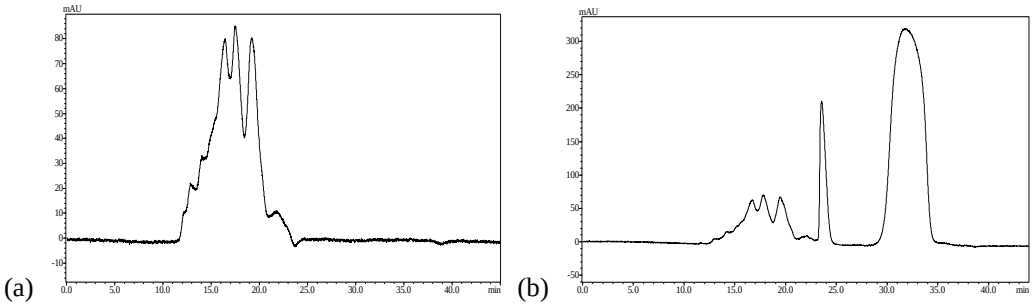

Figure 4. The HPLC spectrum of 0.5h enzymic hydrolysis hydrolysis solustion without the precipitated of the tca (a) and with the precipitated of the tca (b)

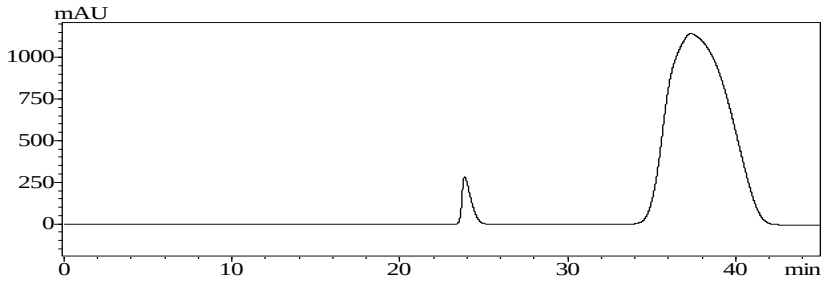

Figure 5. the chromatogram of 10% tca solution at 220 nm

Integrating all the chromatogram get the comprehensive analysis (table 1).It is concluded that enzymolysis liquid of total peak area is increasing as time goes by. More than 3000u peptides area is14.8154%, 7.4609%, 10.7445%, 8.7006% respectively in 0.5 h, 1 h, 2 h, 4h. The rate of this range is gradually decline. The 3000-1000u in the first three is relatively stable at about 41%.The main reason is that both consumption and complement. At 4 h, it fell down to 17.18%.The peak time of 1000 u probably appeared at 17.79 min. The 1000 u tended to be stable in the first 2 hours .Then it began to increase, up to 65.856% at 4.

Table 1. Time-The molecular weight-Square-Precipitation rate

TIME (min) / MWD		30min	1h	2h	4h	The average Precipitation rate
>3000	The peak area	6787157	5911631	5248697	4556613	0.6201
	The peak area(tca)	2038903	2152282	2164943	2017469	
	Precipitation rate	0.699594	0.635924	0.587528	0.557244	
3000-1000	The peak area	1294268	12780259	12766295	5591664	0.3771
	The peak area(tca)	1119716	5950119	5401304	4125829	
	Precipitation rate	0.134865	0.534428	0.576909	0.262146	
<1000	The peak area	7244812	7922166	8946568	19574430	0.2251
	The peak area(tca)	5642828	6614318	6190746	15680459	
	Precipitation rate	0.221122	0.165087	0.308031	0.206351	
Totle area	The peak area	15326241	26614056	26961560	29722707	0.4071
	The peak area(tca)	8801447	14716719	13756993	21823757	
	Precipitation rate	0.4257269	0.447032	0.489755	0.265754	

Tca has different ability of precipitation with the different molecular weight .For more than 3000 u, precipitation rate reached 62.01%.The 3000-1000u is about 37.71%.when the molecular weight less than 1000 u, the rate comes to 22.51%.At the 1, 2 hours, because each component content is stable, tca precipitation rate is respectively 42.57%, 44.70%, 48.98%.Two hours later, forming large numbers of small molecular peptides,

the rate is significantly reduced to 26.58%. For the peak belonging 420 u, because of the addition of tca with some impurities, the precipitation area is bigger. The main reason is that tca have strong absorption peak and a small amount of impurity absorption peak. Thus concluded that tca has different precipitation rate between 9549609u to 420u. In daily life, we thought there is no precipitation rate at 1000u. In fact, less than 1000 u still have slightly precipitation rate.

Conclusion

The digestibility of alkaline protease increased with time. At 4h, enzymolysis achieve stability. The degree of hydrolysis was 16.37%, the peptide yield was 77.30%. The total peak area was still increased. With the extension of reaction time, the proportion of macromolecular peptides decreased and the ratio of small one increased.

Tca played a certain precipitation role of proteins and peptides. For more than 3000 u, the precipitation rate reached 62.01%. 3000-1000u, the rate was about 37.71%, less than 1000u; the rate became 22.51%, mainly concentrated in the 900 u - 1000u. Tca had strong absorption peak in 220 nm.

Acknowledgement

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